AD_____

Award Number: DAMD17-00-1-0249

TITLE: Fox Chase Cancer Center Institutional Breast Cancer Training Program (FCCC-IBCTP)

PRINCIPAL INVESTIGATOR: Jose Russo, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Fox Chase Cancer Center Institutional Breast Cancer Training Program (FCCC-IBCTP)

5. FUNDING NUMBERS
DAMD17-00-1-0249

6. AUTHOR(S)
Jose Russo, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania

E-Mail: J_russo@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The present report covers the work performed by four different trainees working in : *i.- The functional role of chromosomes 11, 13, 14 and 17 in the process of immortalization, transformation and tumorigenesis of human breast epithelial cells*. This study has concluded that the chromosome 17 (pl3.1-13.2) region may contain gene/s controlling programmed cell death, and that the tumorigenic phenotype is associated with alterations at different loci of chromosome 13. *ii.- Lymphedema Prevention in Breast Cancer Survivors*. This study has developed guidelines related to lymphedema prevention among early stage breast cancer survivors to determine the feasibility of the study questionnaire and to determine the most appropriate and effective recruitment approach. *Iii- Roles of γ-synuclein in breast cancer progression and metastasis*. The data indicate that γ-synuclein may promote tumorigenesis by enhancing cell motility through modulating Rho/Rac/Cdc42 and ERK pathways, and promoting cell survival and inhibiting apoptosis through modulating ERK cell survival and JNK-mitochondria-caspase9/3. *iv. Polymorphisms of Human UGT1A6 and UGT1A9 Genes and Functional Differences of the Variant Gene Products*. This study is aimed at defining whether these putative SERM inactivating enzymes have genetic variations that result in predictable phenotypes at both the molecular level and in the patient.

14. SUBJECT TERMS	15. NUMBER OF PAGES
chromosome 17, chromosome 13, apoptosis, γ synuclein, lymphdema, *UGT1A6* and *UGT1A9*, breast cancer	195
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOX CHASE CANCER CENTER INSTITUTIONAL BREAST CANCER TRAINING PROGRAM (FCCC-IBCTP)
JOSE RUSSO, M.D., F.C.A.P., FASCP

Table of Contents

A-INTRODUCTION

The focus of the FCCC Institutional Breast Cancer Training Program (IBCTP) is to integrate the unique talents and interests of the Center's basic scientists, clinical investigators and behavioral scientists to create a comprehensive effort to approach the problems of breast cancer. The rich scientific and intellectual environment of FCCC is nurtured by a cohesive interdisciplinary program that is based on expertise in areas of high relevance to breast cancer. The Institutional Breast Cancer Training Program offers to the postdoctoral trainees practical experience in the fields of cellular and molecular biology, drug resistance and targeted immunotherapy, genetic epidemiology and control, psychosocial and behavioral medicine, as well as breast cancer prevention, diagnosis, and treatment.

B-BODY

B-i. Organization of the FCCC-Institutional Breast Cancer Training Program. Following our statement of work we have accomplished the following tasks:

Task 1. During the second year of the program we have been implementing the training phase of the program by monitoring each of the individual projects (see Section B-ii to v). In addition the trainees have been attending at least one general lecture a week from those offered by the Fox Chase Cancer Center and a special seminar targeted to the formation of the trainees addressing critical subjects in breast cancer (Appendix, Exhibit A). We have established a half-day seminar twice a year in which the trainees present their work in front of the Faculty and the Advisory Panel. The first one took place in March of 2001, the second one on September 2001 and the third one on March of 2002.
Most of the Trainees have presented their work in National and International meeting (see Section D on reportable outcomes). All the Trainees will participate in the Meeting Era of Hope this coming September 25-28 in Orlando Florida.

Task 2. Applicant selection for the second cycle will start this coming July. Due that most of the trainees started on November 2001, the two years cycle will be completed on this time. Using the web page http://www.fccc.edu/postdoc/BreastCaTraining.html developed by our *Recruitment Panel* formed by our Faculty members we have received a numerous requests and applications for this second cycle. Another source is the contacted scientists in the breast cancer area by phone and e-mail. All the applicants were required to present a statement of the fellow's background, training, and professional interests and goals, and a minimum of three recommendation letters. Fellows applying to the program must have a Ph.D. or M.D. degree with background in biology, molecular biology; chemistry, including organic and physical chemistry; mathematics; biochemistry; genetics, and or behavioral sciences. Important evaluation criteria used by *The Recruitment Panel* and the *Applications Evaluation Committee* is the evaluation of personal statements of research and career goals, previous laboratory research experience, publications, and the recommendation letters submitted.

B-ii- Study of the functional role of chromosomes 11, 13, 14 and 17 in the process of immortalization, transformation and tumorigenesis of human breast epithelial cells.

Trainee: Hasan, M. Lareef, M.D.
Mentor: Jose Russo, MD

Introduction

Although the specific causative agent in breast cancer is still elusive, significant advances have been obtained in the biologic and molecular basis of the disease. Genomic alterations such as oncogene activation, loss of genetic material (LOH) and alterations in mismatch repair genes play a major role in the initiation and progression of the disease (1-4). Up to now loss of genetic material has been identified in at least 11 different chromosomes. However, the functionality or significance of these genomic alterations in breast cancer has been poorly understood. The functionality of genomic alterations has been demonstrated by utilizing microcell-mediated chromosome transfer (MMCT), a technique that has been widely used for analyzing the functional role-played by gene/s located on a candidate chromosome (5-8). This technique has allowed mapping putative tumor suppressor genes (9-12), senescence genes (13), and metastasis genes (14-16) to various chromosomes in human cancer cells. To further understand and identify which specific gene/s are involved in the process of immortalization, transformation and tumorigenesis we have developed an *in vitro* experimental system (17-25) that recapitulate the different stages of breast cancer initiation and progression.

Body

a-Transformation of human breast epithelial cells in vitro.

We have shown that the immortalized MCF10-F cells are transformed with the chemical carcinogen benz (a) pyrene (BP) originating the BP1-E cells. The BP1-E cells express *in vitro* phenotypes indicative of neoplastic transformation such as resistant to Fas mediated apoptosis, advantageous growth, anchorage independence, enhanced chemo invasiveness, and absence of ductulogenic capacity (17-26). BP-transformed cells are weakly tumorigenic when transplanted in a heterologous host like the severe compromised immuno deficient mice (SCID). However, when these cells are transfected with the ras oncogene they turn to a highly tumorigenic cell line BP1-T ras (17,18). From these experiments we have developed three cell lines (BP1Tras-T#1, T#2, and T#3) that expressed micro-satellite instability (MSI) and loss of heterozygosity (LOH) in several loci of chromosome 11,13 an 17 (20). In order to test the functionality of these genomic alterations, the chromosome 11, 13 and 17 were transferred to the three cell lines using microcell mediated chromosome transfer technique and cell selection by culture in hygromycin. Chromosome 14 that has been found not to be modified during the process of cell immortalization, transformation, and tumorigenesis was used as control.

b-Genomic changes and functional role.

During the progression of immortalization, transformation and tumorigenesis several genomic changes were detected (18-26). Loss of genetic material in 17q25.3 was associated with the immortalization phenotype and transfer of this piece of chromosome reverted the transformation and immortalization phenotypes (26). In those cells in which retention of chromosome 17p13.1-13.2 were identified the immortalization phenotype was not abrogated but instead they presented reversion of the transformation phenotypes including induced Fas mediated apoptosis in 7 out of the 10 clones studied. The reversion of these phenotypes to the one observed in the MCF10 F cells is associated with retention of an allelic

portion of chromosome 17 at the p13.1-13.2 locus (D17S796). We also have found In the process of transformation by BP the MCF10-F cells loss genetic material in Chromosome 17pI3.1-13.2. The seven clones with chromosome 17 transferred that had retained the portion of 17p are the ones with the abrogated transformation phenotypes. This clearly indicates that this locus harbor gene or genes controlling the Fas complex as well as genes that may be associated with the loss of ductulogenic capacity and anchorage indepent growth.

Colony formation in agar-methocel assay is a technique utilized as an in vitro assay for anchorage independent growth, a parameter indicative of neoplastic transformation. In agar-methocel MCF10-F cells do not produce any colony whereas BP1-E cells produce abundant colony formation with a colony efficiency of 23%. This CE is significantly reduced or abrogated in those clones in which retention of Ch17p13.1-13.2 was found. The clones that did not retain this portion of Ch17 did not change the CE when compared with the parental cells BP1-E. Chromosome 11 transfer is a good control for this study, because it was retained in BP1-E transfected cells but none of the retained portion abrogated the transformation phenotype.

The ductulogenic capacity in collagen matrix is other technique by which we could evaluate the cell's ability to differentiate by producing ductular structures. MCF10F cells form ductules in collagen whereas BP1-E cells loss the ductulogenic capacity. This phenotype was also restored in those clones in which Ch17p13.1-13.2 was retained.

In a previous publication (26) we have shown that transfer of chromosome 17q25.3(D17S785) was associated with abrogation of the transformation and immortalization phenotypes of BP1-E cells. Immortalization is accepted to be an important event in the carcinogenic process (31,32); the immortalization phenomena involve abrogation of cellular program for limiting the rate and the number of cell replications. Elevated levels of telomerase activity have been detected in a number of immortal cell lines and human tumor tissues (33-38). In addition, abrogation of p53 has been associated with immortalization of HBEC (39,40). In the process of spontaneous immortalization of MCF10F cells the following molecular changes have been detected; balance reciprocal translocation 4 (3; 9)(3p13: 9p22) (17), calcium independence for growing in culture (41) insertional mutation of p53 in exon 7 (40) stabilization of telomere length and over expression of H-ferritin (42). These data led us to determine if the telomerase activity and telomere length were affected in the BP1-E cells transferred with Ch. 17 or Ch 11 and we did not find significant difference among the cell lines. On the other hand we did observed significant difference in the response of the cells to apoptosis. We have found that MCF10F and MCF10A cells are sensitive to Fas mediated apoptosis whereas (27) BP1E cells are resistant to Fas mediated apoptosis. The relevance of our work in that the abrogation of Fas mediated apoptosis by Ch. 17p13.1-13.2, is that this locus may contain one or more genes controlling the Fas complex. Fas mediated apoptosis plays a major role in apoptosis and the survival of neoplastic cells (28). We have previously demonstrated that in MCF10F cells Fas as well as Fas ligand increased at mRNA as well as at protein level in comparison to BP1-E cells (Unpublished data). The aberration in the signaling pathways leading to apoptosis may result in cancer, autoimmune diseases and inflammatory disorders (29). In view of this, an understanding of the signaling capabilities of apoptosis-inducing death receptors is essential to understand their roles in cell biology and breast cancer initiation as well as in other organ neoplastic process. In addition, mammary involution is associated with degeneration of the alveolar structure as well as programmed cell death of mammary epithelial cells (27). Estrogen exposure represents the major known risk factor for development of breast cancer in women and estrogen and Tamoxifen regulate Fas/FasL expression has been previously reported (28). Chemotherapeutic drugs such as doxorubicin-induces apoptosis through FasL /Fas signaling pathway in proteolytically cleave FasL in tumor cells and induces cell death (30). All these findings by different investigators indicate that Fas mediated apoptosis in breast cancer plays a major role in breast cancer pathogenesis.

In summary our work demonstrates that the chemical carcinogen benz (a) pyrene induces transformation of MCFl0F cells as evidenced by anchorage independence; loss of ductulogenic capacity in collagen and loss of activation of Fas mediated apoptosis. These transformation phenotypes are associated with LOH in chromosome 17p13.1-13.2 regions (Dl7S796). Transfer of chromosome 17 p13.1-13.2 (D17S796) region reverts the transformation phenotypes as well as Fas mediated apoptosis. We postulate that the chromosome 17 (pl3.1-13.2) (Locus D17S796) region may contain gene/s responsible for maintaining ductulogenic capacity in collagen, colony formation in agar-methocel and controlling programmed cell death through FAS receptor/ligand complex.

c-The role of chromosome 13.

Chromosome 11, 14 and 17 transfer were unable to abrogate the tumorigenic phenotype, therefore the next step was to determine if the changes observed in chromoseme13 were associated with the emergency of this phenotype. Chromosome 13 transfer to BP1 Tras T #1, T#2, and T #3 have allowed us to obtain several clones. Of interest was the observation that those clones derived after transfer of chromosome 13 to T# 1 became senescent in passage 1, whereas T#2 transfected became senescent by passage 5- 6

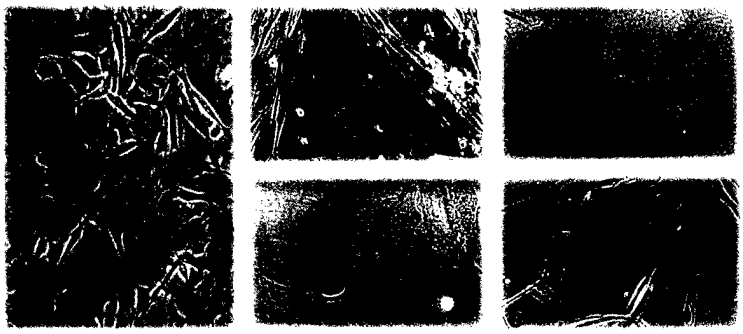


Figure 1: a- BP1 T ras T#2 cells in culture prior to transfer of chromosome 13; b, c, d and e show clones 1,2,3 and 4 respectively, at passage five. All these clones are arrested in grow and entering in senescence.

(Figure 1). A different result was obtained when the BP1-Tra-T#3 was transferred with chromosome 13 in which several permanent cell lines were originated. They were designated BP1 T ras T#3-13 hygro I2# 1(clone 1), BP1 T ras T#3-13 hygro I2# 2(clone 2), BP1 T ras T#3-13 hygro I2# 3(clone 3), BP1 T ras T#3-13 hygro I2#4(clone 4), BP1 T ras T#3-13 hygro I3# 1(clone 5), BP1 T ras T#3-13 hygro I3# 2(clone 6), BP1 T ras T#3-13 hygro I3# 3(clone 7) and BP1 T ras T#3-13 hygro I3#4(clone 8).

Whereas, MCF-10F cells grew forming a monolayer of polyhedral cells without overlapping as previously described (26), BP1-T ras cells grew faster and have a tendency to overlap. MCF-l0F cells do not form colonies in agar methocel, form ductules in collagen matrix and do not induce tumorigenesis in

SCID mice, instead BP1-Tras cells grew in agar-methocel and produce colonies and induced tumorigenesis in SCID mice. As an *in vitro* indication of neoplastic transformation we studied colony formation in agar methocel and ductulogenesis in collagen matrix in parental cells as well as chromosome 13 transfected cell lines. We did not identify any significant differences in these two assays between chromosome 13 transfected clones and parental tumorigenic cells except clone 6 that had a reduced colony efficiency and smaller colony size in comparison to other clones and parental cells (Figures 2A-D). We calculated colony efficiency, colony size, plating efficiency and survival efficiency as previously reported(26) . Plating efficiency (PE) is calculated as number of viable cells in each plate after 24 hrs of plating. Figure 2A indicates the parental cells as well as all clones had similar PE. Colony efficiency is calculated as percentage of colonies after 21 days in comparison to total number of viable cells after 24 hrs (Figure 2B). Colonies which are more than 50 micron were counted and measured by using microscopic grid and calculated mean colony size (CS) (Figure 2C). Survival efficiency (SE) was calculated as percentage of number of viable cells after 21days without forming colonies in comparison to number of viable cells after 24 hrs.

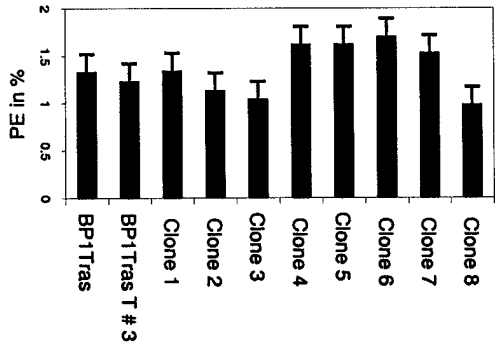


Figure 2A: Plating efficiency, of chromosome 13 transfected clones.

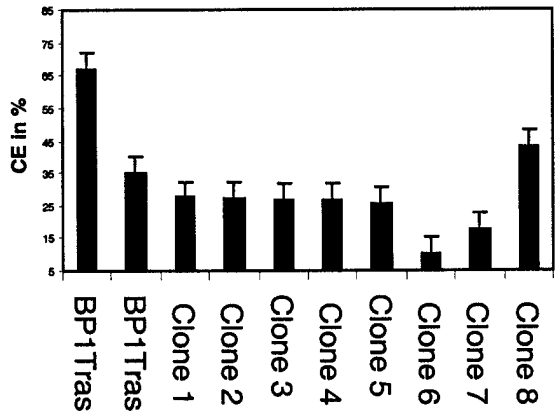


Figure 2B. Colony efficiency, of chromosome 13 transfected clones

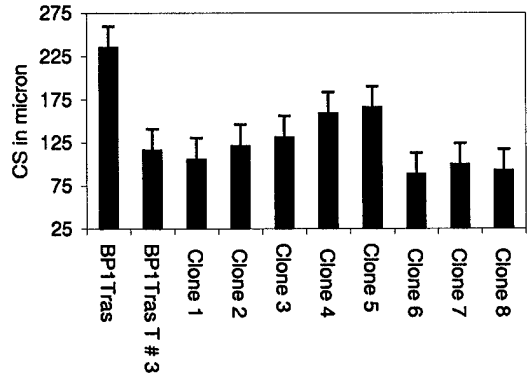


Figure 2C: Colony size, of chromosome 13 transfected clones.

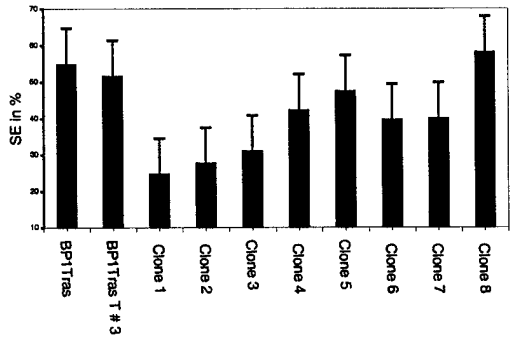


Figure2D: Survival efficiency, of chromosome 13 transfected clones

d-The abrogation of the tumorigenic phenotype by Chromosome 13.

For testing this phenotype we have inoculated $15x10^6$ cells of clones 1,2, 4, 5 and 6 in five SCID mice per clone. Chromosome 13 transfected cell lines either fails to produce tumor or tumors were very small in comparison to parental tumor cell line (Figure 3).

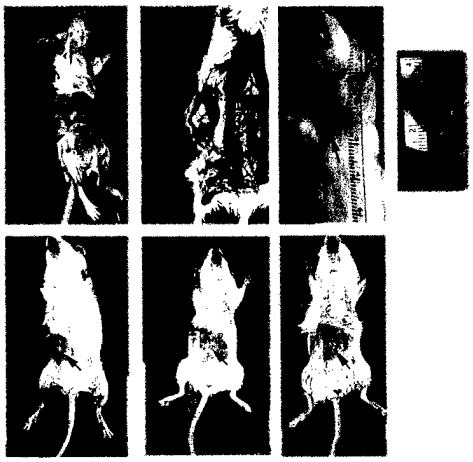


Figure 3: a, b and c tumors following inoculation of parental BP1 T ras T#3 cells. d, e and f tumors induced by inoculation of clones 2,4 and 8, that are transferred with chromosome 13

Histological examination showed that parental cells produced very malignant poorly differentiated carcinomas, whereas chr13 transfected clones indicated either very small spindle cell tumors or foreign body reaction.

We have repeated this experiment by using eight clones instead of four and found that BP1-Tras and BP1-Tras T#3 (parental control) cells produced poorly differentiated tumors, whereas chromosome 13 transfected cells either failed to produce tumors or they were much smaller than parental cells produced tumor (Figure 4). Histologically they are small spindle cells tumors. Chromosome 14 transfected BP1 T ras T#3 cells did not abrogate tumor

formation All five clones with chr14 produced large tumors in SCID mice like parental BP1T ras and BP1 T ras T#3 parental cells (Figure 4).

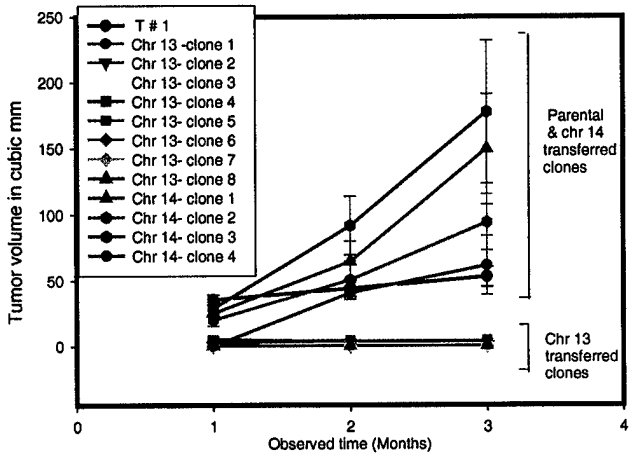


Figure 4: Tumorigenic response. Volume of tumors from parental Bp1 T ras t#3 cells and from clones transferred with chromosome 13 and chromosome 14.

In summary, transfer of BP1E-Tras cells produce tumors in SCID mice and transfer of chromosome 13 abrogate the tumorigenesis. Chromosome 14 does not play any significant role in cell transformation and immortalization in human breast epithelial cells.

B-iii. Lymphedema Prevention in Breast Cancer Survivors (Project 1) and Knowledge About Genetic Risk Assessment and Interest in Genetic Risk Assessment for Hereditary Breast/Ovarian Cancer Among Inner City Women (Project 2). By: Kerry Sherman, Ph.D.

Trainee: Kerry Sherman, Ph.D.
Mentor: Suzanne Miller, Ph.D.

Project 1:

Introduction

Approximately 20-30% of women develop lymphedema (*LE*) following breast cancer treatment; this condition has been associated with psychological distress and diminished quality of life. Effective symptom management requires that women not only recognize early signs of this condition, but that they uptake and maintain precautionary practices over their lifetime. Yet, the limited data available indicate that knowledge and use of symptom minimization precautions are poor, particularly over time. Unfortunately, little is known about how breast cancer survivors perceive their *LE* risk status, and the cognitive-affective factors that promote the uptake of, and adherence to, *LE* symptom minimization precautions. Further, the moderating role of individual differences in attentional style (i.e., *high-monitoring*, which entails attending to, and amplifying health threats, versus *low-monitoring,* which entails distracting from and ignoring such threatening cues) has not been explored.

Body

Guided by the Cognitive-Social Health Information Processing (C-SHIP) model, we will conduct a longitudinal study, to assess the barriers and facilitators associated with knowledge about, and initiation and sustained adherence to, *LE* symptom-minimization practices among breast cancer survivors currently unaffected by *LE*. We will explore the mediating role of cognitive–affective variables, and the moderating role of attentional style, on knowledge, uptake and adherence over time. Toward this end, we will survey levels of knowledge, and the practice of symptom minimization precautions at baseline, and again at 6-, and 12-month follow-up. **Specific Aims. Aim 1: To delineate the underlying cognitive-affective mediating mechanisms promoting the uptake of *LE* symptom-minimization practices, and their sustained adherence, over time.** Guided by the C-SHIP model, we predict that a woman's pattern of cognitive-affective processing dynamics (i.e., risk perceptions, expectancies, affect and self-regulatory strategies) will impact on her *LE*-knowledge, and uptake of, and adherence to, symptom minimization practices. Specifically, greater *LE*-knowledge, greater intent to establish practices and/or adhere to existing practices, as well as greater uptake of recommendations and sustained adherence will be associated with heightened risk perceptions; greater self-efficacy, greater perceived benefits of, and fewer barriers to, enacting symptom minimization practices; lower *LE*-related distress; and greater ability to perform self-regulatory strategies, over time. **Aim 2: To assess the moderating role of attentional style on the uptake of and adherence to, *LE* symptom minimization practices, over time.** We predict that high monitors will be characterized by heightened perceived *LE*-risk. This focus on risk will also activate increased risk-related distress, lower self-efficacy, and fewer perceived benefits of, and greater barriers to, the uptake of *LE*-precautions. Hence, high monitors will consistently display greater *LE*-related knowledge, and greater intent to adhere to existing practices, and to establish practices, over time, than low monitors, whose level of intent will diminish over time. However, high monitors' focus on threat may undermine their intent to adhere to recommended behaviors; ultimately high monitors may be as non-adherent as low monitors. Thus, we predict that there will be no differences in actual uptake and adherence between high and low monitors, over time. **Study Design.** Women treated for breast cancer,

who are presently *LE*-symptom free (N=178), will complete a baseline questionnaire on the day of their post-adjuvant therapy follow-up appointment. Cognitive-affective mediators (e.g., perceived *LE* risk, expectancies and beliefs, risk-related distress, self-regulatory strategies) will be assessed at baseline, and at 6-, and 12-month follow-ups. We are currently awaiting DOD IRB approval for this study.

Project 2

Introduction

Among minority women in the United States, the incidence and mortality rates for breast cancer are disproportionately high compared with that of Caucasian women. The recent discovery and identification of the *BRCA1* and *2* genes associated with inherited susceptibility to breast and ovarian cancer has led to the implementation of breast cancer familial risk assessment programs. *BRCA1/2* mutations account for approximately 5% of breast cancer cases, and confer an estimated range from 36-85% lifetime risk of developing breast cancer, and a 16-60% lifetime risk for ovarian cancer. Recent investigations of *BRCA1/2* mutations in African American populations have identified similar rates of these mutations as have been previously identified in Caucasian populations. Interest in, and uptake of, breast and ovarian cancer risk assessment and genetic testing programs are especially low for minority women and women of low socio-economic status. However, since most of the research about the uptake and impact of risk assessment programs has focused on Caucasian women, few data are available about how minority women understand their genetic risk status, and the psychosocial factors that promote or undermine participation in genetic risk assessment programs. Lack of sufficient knowledge of available surveillance regimens and risk assessment programs may be one factor related to underutilization of breast cancer screening and risk assessment resources by low-income women, since women who lack sufficient knowledge of available surveillance regimens cannot take advantage of them (43,44).

Body

Guided by the Cognitive-Social Health Information Processing (C-SHIP) framework, we are conducting a cross-sectional survey, to explore levels of knowledge about *BRCA1/2* genetic mutations in relation to breast and ovarian cancer susceptibility, and level of interest in receiving cancer risk counseling and *BRCA1/2* genetic testing among inner city women. Psychosocial predictors assessed include perceived risk of developing breast/ovarian cancer, perceived control over cancer, breast/ovarian cancer risk-related intrusive and avoidant ideation (Revised Impact of Events Scale), fatalism (Powe Fatalism Scale) and social support (Donald & Ware Social Contact and Group Participation Scale). Participants (N=78) recruited from two gynecological clinics at Temple University Hospital were moderately well-educated (39.2% completed high school, 35.5% college educated), average age 40 years, and comprised predominantly minority ethnicities (15.4% Hispanic, African American 37.2%, Asian/ Pacific Islander 6.4%, Caucasian 33.3%). Level of genetics knowledge was strikingly low with 86% of participants reporting that they had never heard of BRCA1/2 genes. In contrast, more than one half of the participants indicated a high level of interest to pursue cancer risk assessment counseling for breast/ovarian cancer (68.5%) and to receive genetic testing (63.6%). Interest in risk assessment was associated with increased perceived risk for ovarian cancer ($p<.05$), increased perceived control over ovarian cancer ($p<.05$), and enhanced social support ($p<.01$). In addition, greater social support was associated with increased interest in undergoing genetic testing ($p<.01$).

Discussion.

These interim results highlight the importance of the woman's perceptions and her beliefs related to breast/ovarian cancer when contemplating participation in genetic risk assessment programs. The finding that heightened perceived risk and greater social support was associated with increased interest in pursuing breast/ovarian cancer risk assessment is consistent with similar studies among African American women. Accuracy of risk assessment may be improved through educational intervention, and, when risk information is provided within the context of a trusting relationship (i.e., social support), interest in genetic testing may increase. Moreover, the association between perceptions of control over ovarian cancer and level of interest in genetic risk assessment extends the existing knowledge base that has shown a strong link between participation in cancer screening and beliefs in the efficacy of cancer control approaches. However, there appears to be a large discrepancy between interest levels and the accompanying knowledge base. In this study, high levels of interest were evident within a poorly informed population. These preliminary results build upon those of Hughes et al. (45) who showed that despite having lower levels of knowledge, African American women report more positive attitudes about the benefits of genetic risk assessment. Given the complexity and uncertainty of genetic risk information, these preliminary results suggest the need for tailored educational and counseling risk assessment protocols, targeted to an inner city population. Data collection for this project is ongoing, with a target sample size of 100.

B-iv- Roles of γ-synuclein in breast cancer progression and metastasis.

Trainee: Zhong-Zong Pan, Ph.D.
Mentor: Andrew Godwin, Ph.D.

Introduction

The synucleins (α, β, γ, synoretin) are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Little is known about the normal functions of synucleins in physiological conditions. Of the synucleins, α-synuclein is the best characterized because of its potential significance in neurodegenerative diseases including Parkinson's Disease. Recently we and others have found that γ-synuclein is dramatically up-regulated in the vast majority of late-stage breast (70%) and ovarian (> 85%) cancers and that γ-synuclein over-expression can enhance tumorigenicity (46-48). We also observed that expression of γ-synuclein induces a phenotype similar to that induced by activation of RhoA/Rac/CDC42, altering the appearance of focal adhesions and stress fibers, and enhancing motility and invasion in ovarian cancer cells. Recent studies by Ji and colleagues have also shown that when γ-synuclein is overexpressed in a breast tumor derived cell line, the cells experience a dramatic augmentation in their capacity to metastasize *in vivo* (49). Based on these known data, we hypothesized that γ-synuclein may be a proto-oncogene, and that the abnormal expression of this protein (i.e., oncogenic form) in breast and ovarian tumors may contribute to the metastatic spread and high morbidity associated with advance stages of these diseases. To address this hypothesis, we first ectopically over-expressed γ-synuclein in several cancer cell lines and the effects of γ-synuclein on the phenotypes of these cells were charaterized. The mechanisms underlying γ-synuclein induced cell phenotype changes were investigated by biochemical and cellular assays. We found that γ-synuclein is associated with two major mitogen-activated kinases (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1). Over-expression of γ-synuclein may enhance cell motility by activating Rho/Rac/Cdc42 and ERK pathways. JNK activation induced by stress (UV, arsenate, and heat shock) and chemotherapy drugs (Taxol, vinblastine) can also be down-regulated by γ-synuclein. Finally, we found that γ-synuclein can protect chemotherapy drug-induced cell death by modulating the ERK cell survival pathway and the JNK-mitochondria-Caspase9/3 pathway.

Body

γ-Synuclein interacts with ERK and JNK MAP kinases in cancer cells - α-Synuclein has recently been reported to bind directly to the ERK2 kinase (50). Therefore, we evaluated whether γ-synuclein could also interact with the ERK kinases as well as other MAPKs. By co-immunoprecipitation

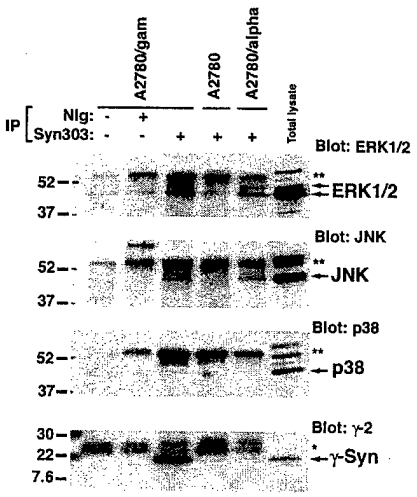


Fig. 1. Interaction between γ- and α-synucleins with ERK1/2 and JNK. Cell lysate from A2780, A2780/gam or A2780/alpha were immunoprecipitated with Syn303, NIg (normal IgG) or irrelevant antibodies (not shown here). The proteins in the immunoprecipitates were identified by immunoblotting with antibodies against ERK1/2, JNK1, p38, and γ-2 (a polyclonal antibody specific for γ-synuclein). Molecular mass standards (in kilodaltons) are indicated on the left. Non-specific bands around the IgG heavy (**) and light (*) chains are indicated by asterisks.

approaches, we were able to demonstrate a novel association of γ-synucleins with ERK1/2 and JNK1 kinase, but not with the p38 kinase (**Fig. 1**). We also confirmed that α-synuclein is associated with ERK1/2 as well with JNK1 (**Fig. 1**), which is consistent with the recently studies using neuro2a, a neuronal cell line (51). These data indicate that γ- and α-synuclein can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.

Elevated activity of ERK in cells over-expressing γ-synuclein - We next evaluated whether these protein interactions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ-synuclein, the activated ERK1/2 was increased 2-3 fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (**Fig. 2**). In contrast, α- and β-synucleins appeared to have little or no effect on the activity of ERK1/2 (**Fig. 2A**) although α-synuclein was also found to be associated with ERK (as described above and shown in **Fig. 1**) in A2780 cells. In HEK 293 cells, the basal level of ERK activation is undetectable and γ-synuclein over-expression does not increase its activation level (**Fig. 2B**). Structural analysis indicate that γ-synuclein does not contain any kinase domain, suggesting that the activation of ERK is mediated by other kinase. Since MEK1/2 is required for the activation of ERK1/2 in response to many mitogens, we determined whether MEK1/2 is still required

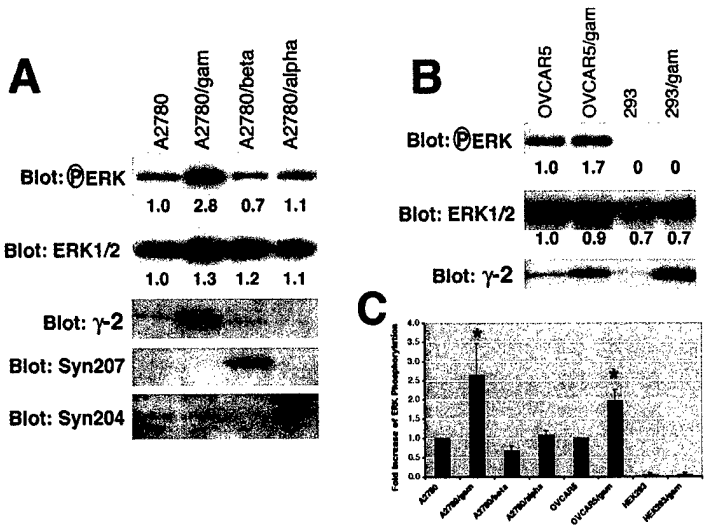


Fig. 2. Activation of ERK in cells over-expressing γ-synuclein. A, ERK1/2 activation is enhanced in γ-synuclein over-expressing A2780 cells. Whole cell lysates (40 μg/lane) from A2780 cells (parent) or A2780 cells transfected with γ-, β-, or α-synuclein were separated by SDS-PAGE and blotted with appropriate antibodies. The levels of activated ERK or total ERK1/2 were determined using an anti-phospho-ERK specific antibody or ERK1/2 antibodies, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band (the integrated densitometry reading number of A2780 or OVCAR5 was assigned to an arbitrary unit 1.0, and the other readings were normalized thereafter). The synucleins were evaluated by blotting with specific antibodies, i.e., γ-2 for γ-synuclein, Syn207 for β-synuclein, and Syn204 for α-synuclein, respectively. B, activation of ERK by γ-synuclein in OVCAR5 cells but not HEK 293 cells. Whole cell lysate (40 μg/lane) from parental or γ-synuclein over-expressing cells were separated and blotted as in A. Panels A and B are representative of at least three independent experiments with comparable results. C, fold increase of ERK activation. The data shown are the average ± S.E. of three independent experiments. Phosphorylated ERK was normalized to the protein level of total ERK. The basal levels of ERK phosphorylation in the parental A2780 or OVCAR5 cells were set as 1.0. (*) Represents significant difference compared to the parental cells ($p < 0.05$).

for γ-synuclein mediated activation of ERK1/2. When cells over-expressing γ-synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (**Fig. 3A**). We further studied the relation of γ-synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ-synuclein and ERK1/2 was still present (**Fig. 3B**). These data indicate that γ-synuclein may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ-synuclein.

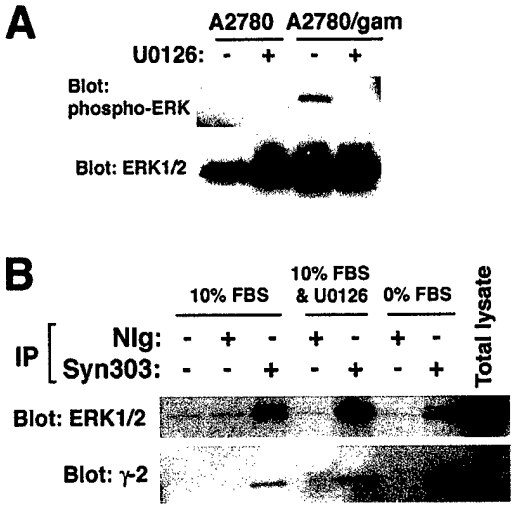


Fig. 3. Requirement of MEK1/2 for γ-synuclein enhanced ERK1/2 activation. A, A2780 and A2780/gam cells untreated or treated with the MEK1/2 inhibitor, U0126 (10 μM), were lysed and 30 μg of proteins were loaded into each lane. As in Fig. 2, anti- phospho-ERK1/2 specific antibody was used to detect activated ERK1/2; and the antibody against ERK1/2 was used to detect the protein level of total ERK1/2. B, the interaction between ERK and γ-synuclein is independent of the activation status of ERK1/2. A2780/gam cells in normal 10% FBS medium, 10% FBS medium with U0126 (10 μM), or serum-free medium were lysed and immunoprecipitated with Syn303 or control IgG. The proteins in the immunoprecipitates were detected with the antibodies against ERK1/2 or against human γ-synuclein (γ-2). The autoradiogram shown is the representative of three independent experiments with comparable results.

Over-exprssion of γ-synuclein leads to increased cell motility- Recent studies by Jia and colleagues indicated that γ-synuclein may increase cell migration and metastasis (49). We also established several stable cancer cell lines that over-express γ-synuclein (Fig. 4A, and data not shown) and found that γ-synuclein can enhance cell motility as analyzed by Boyden chamber assay (Fig. 4B, 4C, 4D, and data not shown). Consistent with their role in cell migration, more stress fibers were found in cells over-expressing γ-synuclein (Fig. 5)

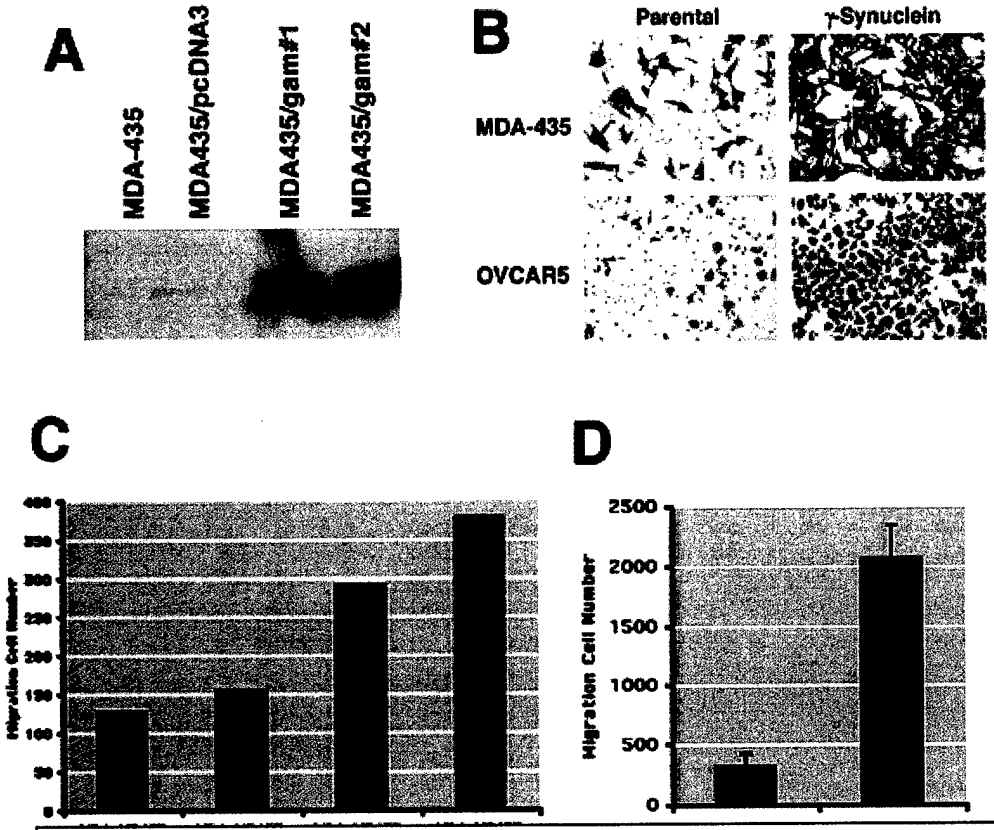


Fig.4. γ-Synuclein enhance cell migration. A, γ-synuclein expression in parental MDA-MB-435 cells, and those stably transfected with pcDNA3 vector alone, or with CMV-γ-synuclein (two clones were shown here). B throuhg D, Boyden chamber assay for cell migration. Cells migrated to the lower chamber were stained (B) and counted (C, D).

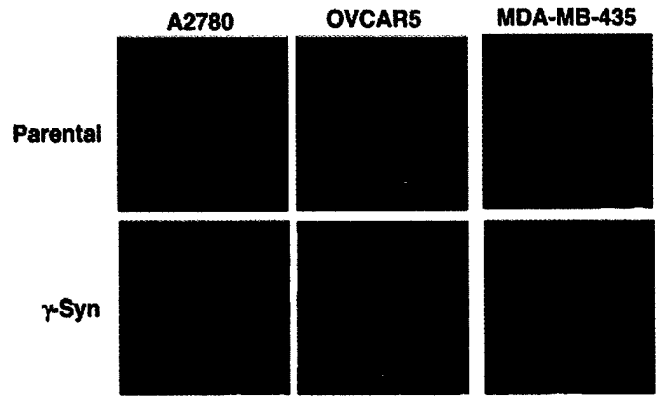


Fig. 5. Induction of stress fibers by γ-synuclein. Parental cells (A2780, OVCAR5, and MDA-MB-435) and their corresponding γ-synuclein over-expressing cells were stained with Rhodamin-palloidin. Photography was taken using confocal microscope at FCCC image facility.

Over-exprssion of γ-synuclein leads to activation of at least one member of the Rho family GTPase- Cell migration and invasion involves a series of coordinated complex processes, including focal adhesion formation in the front and release of adhesion in the back, polarized stress fiber formation and disassembly and contraction (52). Several protein kinases are known to regulate these processes, including Rho/Rac/Cdc42 small GTPase proteins, ERK, Crk, and Akt (53-56). Among these effector kinases, Rho family members play a pivotal role in regulating stress fiber and focal adhesion formation. In cells over-expressing γ-synuclein, we found at least one major Rho/Rac/Cdc42 member is activated although the protein levels of these proteins were not affected (Fig. 6).

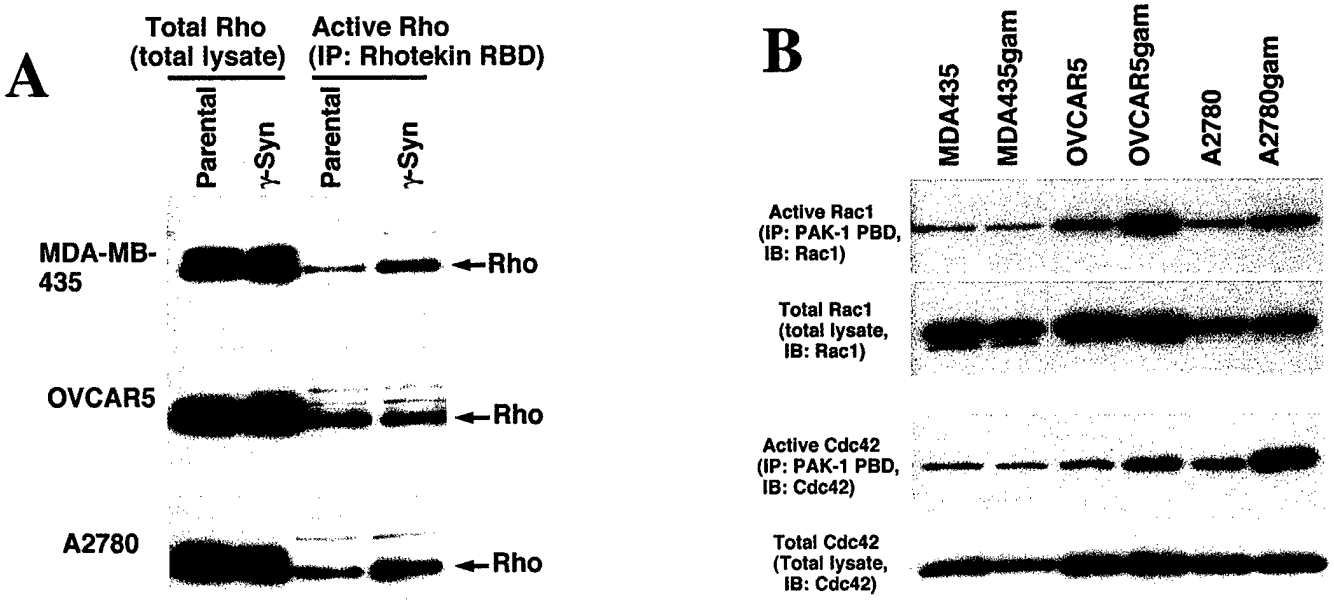


Fig. 6. Enhanced activation of Rho (A) and Rac/Cdc42 (B) in cancer cells over-expressing γ-synuclein. A, whol3 cell lysate (2 mg protein) were immunoprecipitated with Rhotekin Rho binding domain and analyzed by Western blot with anti-Rho antibody. B, who cell lysate (2 mg protein) were immunoprecipitated with PAK1 PBD and analyzed by Western blot with anti-Rac and anti-Cdc42 antibodies, respectively.

Requirements of Rho/Rac/Cdc42 and ERK for γ-synuclein-enhanced cell motility-
As described above, over-expression of γ-synuclein also leads to enhanced activation of ERK. To determine whether and to what extent Rho/Rac/Cdc42 or ERK kinase contribute to cell motility and invasiveness, parental cancer cells and their corresponding γ-synuclein over-expressing cells were treated with specific inhibitors. When treated with *C. difficile* toxin B which can inactivate most, if not all, members of the Rho family, and U0126, an inhibitor of ERK activation, the cell migration in both parental and γ-synuclein over-expressing cells were almost completely blocked (Fig. 7). These data indicate that both Rho and ERK pathways are involved in the parental basal level cell migration and the enhanced cell migration induced by γ-synuclein.

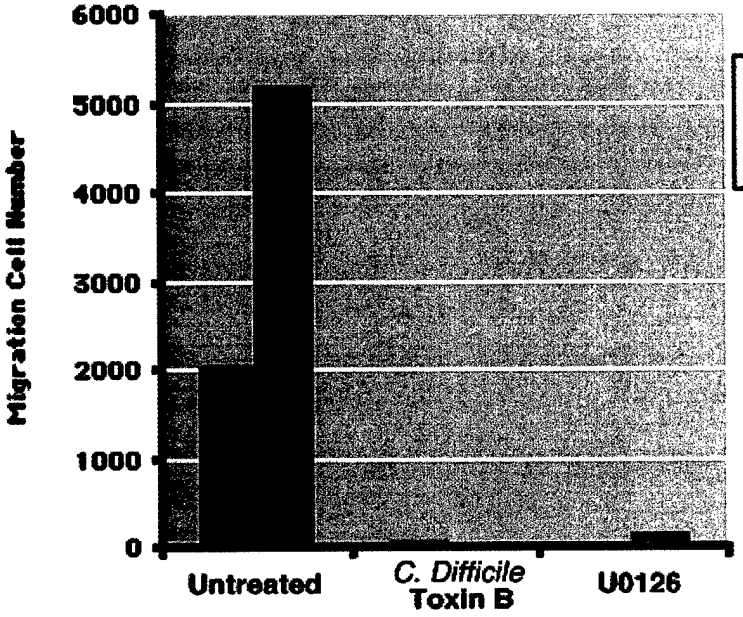


Fig. 7. Blockage of cell migration by Rho and ERK inhibitors. Parental cells or γ-synuclein over-expressing cells were allowed to attah for 8hr before treated with *C. difficile* Toxin B (0.5 ng/ml) or U0126 (10 μM) before analysis of migrated cells at 52hr.

Down-regulation of JNK activation by γ-synuclein in response to UV - JNK is activated by stress signals including UV which leads to mitochondria mediated apoptosis (57). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ-synuclein was over-expressed or not (**Fig. 8**). JNK was highly activated in the parental cells

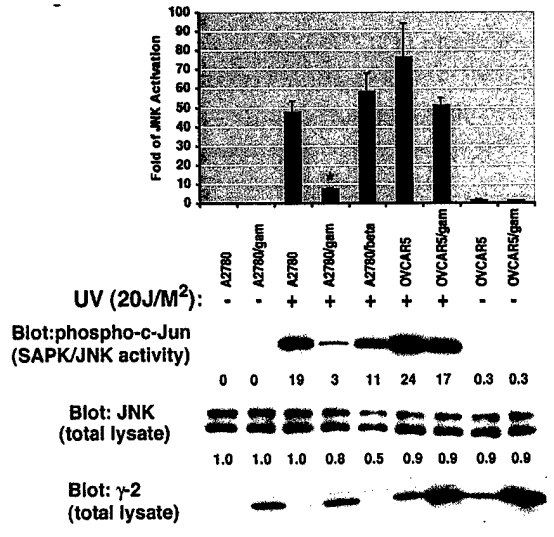


Fig. 8. Inhibition of JNK activation by γ-synuclein in response to UV treatment. A2780, A2780/gam, A2780/beta, OVCAR5, OVCAR5/gam cells were un-treated or treated with UV (20 J/M^2) and cells were lysed at 30 min. JNK activities were analyzed by an immunocomplex kinase assay using GST-c-JUN as substrate. The phosphorylated GST-c-JUN by activated JNK was evaluated by immunoblotting with anti-phospho-c-JUN specific antibody. The protein levels of JNK and γ-synuclein were determined by immunoblotting with anti-JNK and γ-2 antibody, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. (*) Represents significant inhibition of JNK activation compared to that in the parental cells ($p < 0.05$).

when treated with UV (**Fig. 8**). In cells over-expressing γ-synuclein, the activation of JNK was almost completely blocked in A2780/gam cells ($p < 0.05$) and was down-regulated by approximately 50% in OVCAR5/gam cells when treated with UV (**Fig. 8**) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ-synuclein specific since over-expression of α- and β-synucleins appeared to have little or no effect on the activation of JNK by UV (**Fig. 8**, and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific, or it could be the high endogenous γ-synuclein expression in OVCAR5 cells. Collectively, these data indicate that stress-induced activation of JNK can be blocked by γ-synuclein over-expression in a variety of cell lines.

γ-Synuclein may protect paclitaxel (Taxol) induced cell death by regulating JNK and ERK activities - Based on the data presented above, we hypothesized that γ-synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated γ-synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, cell survival and cell death are regulated by the counter-balance between the survival factors and the apoptotic signaling pathways. Since UV treatment also activates the cell survival pathways ERK [(58), and data not shown] and PI3K-AKT (59,60), the initiation of the mitochondria associated caspase pathway may be blocked by the activation of ERK or AKT in A2780 cells. In support of these findings we did not observe the cleavage of the caspase-3 substrate PARP in A2780 cells when treated with UV (data not shown).

We next evaluated the survival of γ-synuclein over-expressing cells in response to Taxol, a commonly used chemotherapeutic drug. In addition to its role in affecting microtubule assembly, Taxol is known to lead to apoptosis via the mitochondria by activating the JNK signaling pathway and Taxol-induced apoptosis can be enhanced by MEK inhibition (61-64). In A2780 cells, Taxol did not affect the basal level activity of ERK or the activation of ERK by γ-synuclein (**Fig. 9A**). To test the effect of γ-synuclein on cell survival, cells were treated with Taxol for varying lengths of time. At 48hr after treatment, 45-60% of A2780 cells had died, while only 7-15% of A2780/gam cells were dead indicating that Taxol induced cell death can be suppressed by γ-synuclein over-expression (**Fig. 9B**). When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced

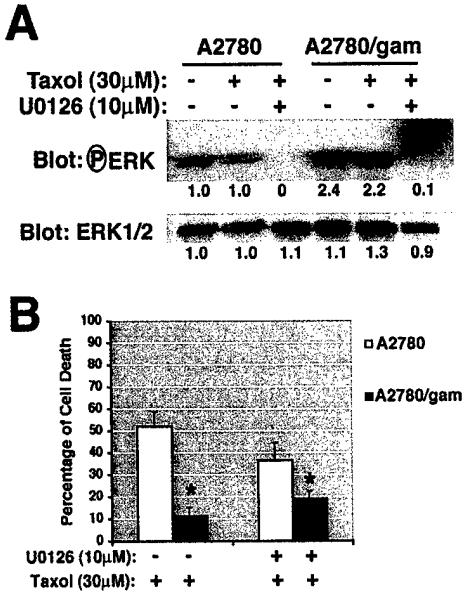


Fig. 9. γ-Synuclein protects cells from paclitaxel (Taxol) induced cell death and is partially mediated by ERK activation. A, Taxol does not affect ERK activity or γ-synuclein mediated ERK activation. A2780 or A2780/gam cells pre-treated with or without U0126 (10 μM) were treated with Taxol (30 μM) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **B,** cell death induced by Taxol was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with Taxol for 48hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$).

by ~25% in A2780 cells but was nearly doubled in A2780/gam cells (**Fig. 9B**). These data suggest that enhanced cell survival in γ-synuclein over-expressing cells is partially mediated by activation of ERK.

To determine whether the protective role of γ-synuclein on cell survival is also mediated through down-regulating JNK associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (61,63), JNK was activated in A2780 cells when treated with 30 μM Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol ($p < 0.05$) (**Fig. 10A**). In the parental A2780 cells, caspase-3 was highly activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in γ-synuclein over-expressing ovarian cancer cells ($p < 0.05$) (**Fig. 10B**). These data indicate that Taxol activated JNK mediated caspase apoptotic pathway was significantly attenuated in cells over-expressing γ-synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by γ-synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

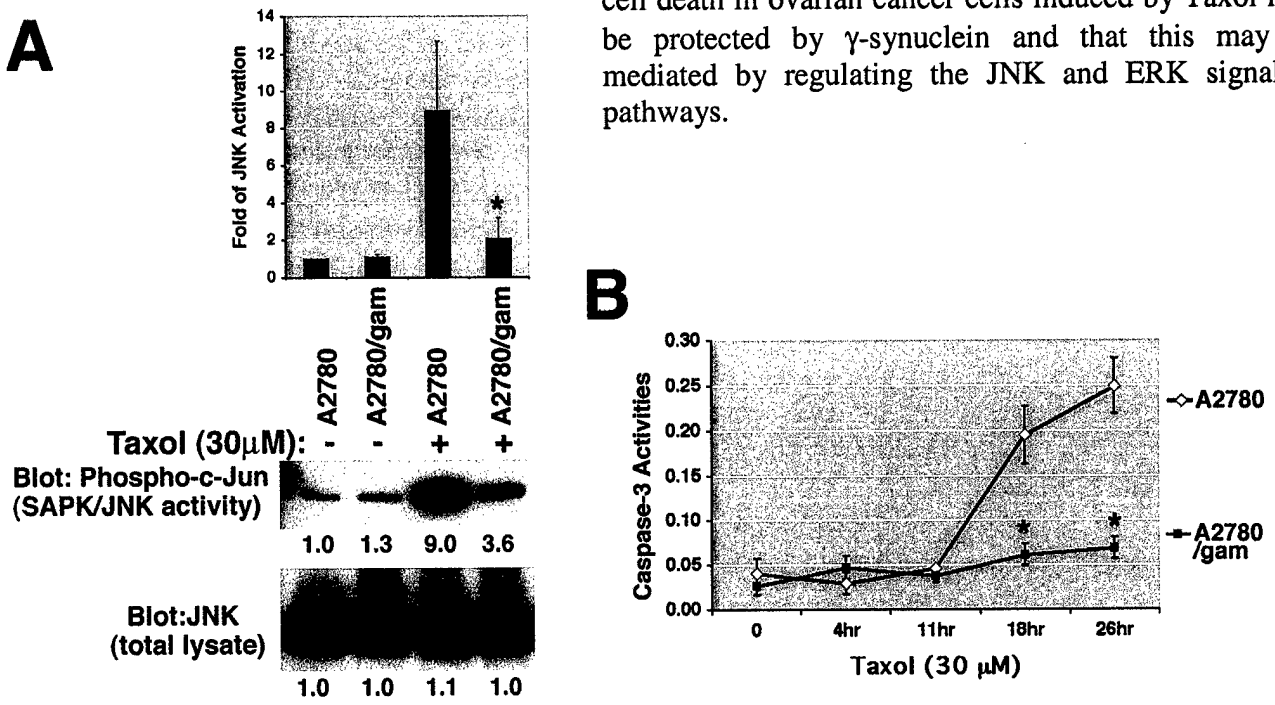


Fig. 10. Taxol activated JNK and caspase-3 apoptotic pathway is blocked by γ-synuclein. A, down-regulation of JNK activation by Taxol in cells over-expressing γ-synuclein. Cell lysates from A2780 and A2780/gam cells treated with or without Taxol (30 μM) for 60 min were assayed for JNK activity (see the legend for **Fig. 8** for experimental details). The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. **B,** down-regulation of caspase-3 activity activated by Taxol in A2780/gam cells. A2780 and A2780/gam cells treated with Taxol (30 μM) for different time lengths were lysed, and approximately 20 μg protein were incubated with the caspase-3 substrate Ac-DEVD-pNA for 4hr at 37°C. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate reader. The graph represents the average ± S.E. of three independent experiments. In panels A and B, (*) represents significant difference compared to that in the parental cells ($p < 0.05$).

γ-Synuclein over-expression leads to protection from vinblastine but not etoposide induced cell death – To demonstrate whether the effects of γ-synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents; Taxol binds to microtubule polymers while vinblastine binds to monomers and dimers. When treated with vinblastine (either 0.1, 1.0, or 10 μM for 30 hr), cell death in A2780/gam cells was significantly lower ($p < 0.05$ for all the three concentrations tested) as compared to the parental cells (**Fig. 11A**). Consistent with other studies using a variety of tumor cell lines (63-67), vinblastine strongly activate JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by γ-synuclein over-expression (**Fig. 11B**). Furthermore, we observe that treatment with vinblastine results in a two-fold increase in phosphorylated ERK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (**Fig. 11B**). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (**Fig. 11A & C**). These data indicate that suppression of vinblastine-induced cell death by γ-synuclein may be mediated by inhibition of JNK activation.

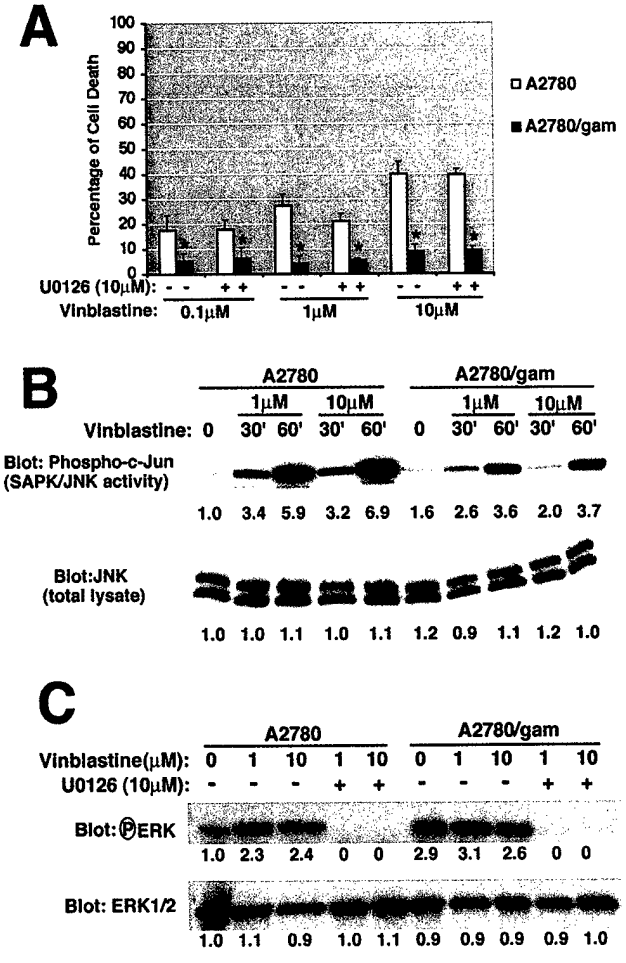


Fig. 11. Vinblastine induced cell death and activation of the MAPK pathways in γ-synuclein over-expressing cells. A, the cell death induced by vinblastine was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with vinblastine (0.1, 1, or 10 μM) for 30 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$). **B,** inhibition of JNK activation by γ-synuclein in response to vinblastine treatment. A2780 and A2780/gam cells were un-treated or treated with vinblastine (1μM, and 10μM) for 30 min and 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in the legend for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C,** the effect of vinblastine on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with vinblastine (1 μM and 10 μM) in the absence or presence of U0126 (10 μM) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (66-69). When treated with 1, 10, or 100 μM of etoposide for 56 hrs, there was no significant difference in cell survival between A2780 and A2780/gam cells (**Fig. 12A**). As might be

predicted, JNK was not activated in response to etoposide treatment (**Fig. 12B**). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down-regulated within 30 min of treatment with 10 or 100 μM of etoposide (**Fig. 12C**). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and γ-synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.

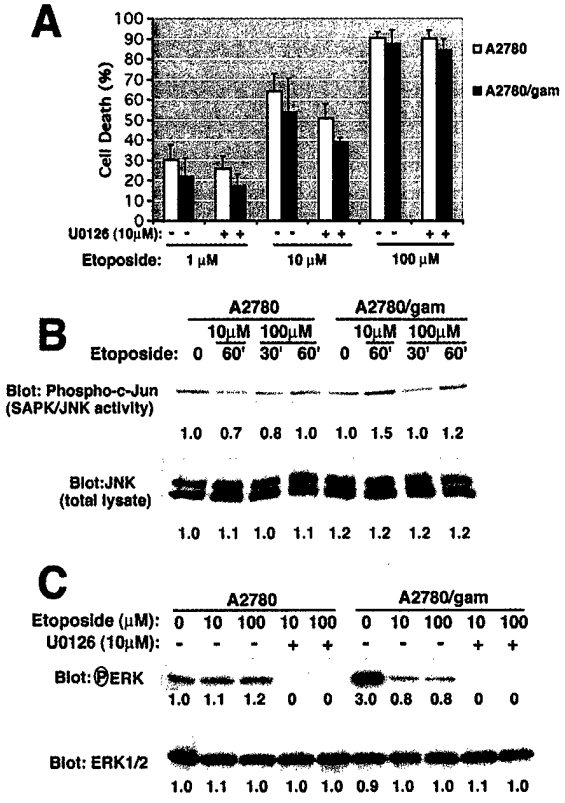


Fig. 12. Effects of γ-synuclein over-expression on etoposide induced cell death and activation of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express γ-synuclein. A2780 and A2780/gam cells treated with etoposide (1 μM, 10 μM, 100 μM) for 56 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. B, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 μM, and 100 μM) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. C, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 μM and 100 μM) in the absence or presence of U0126 (10 μM) for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results.

Summary. Based on the data we presented above, we proposed the following model (Fig. 13) that γ-synuclein may enhance cell migration and metastasis, promote cell survival and inhibit apoptosis in tumor progression by modulating Rho and MAPK pathways.

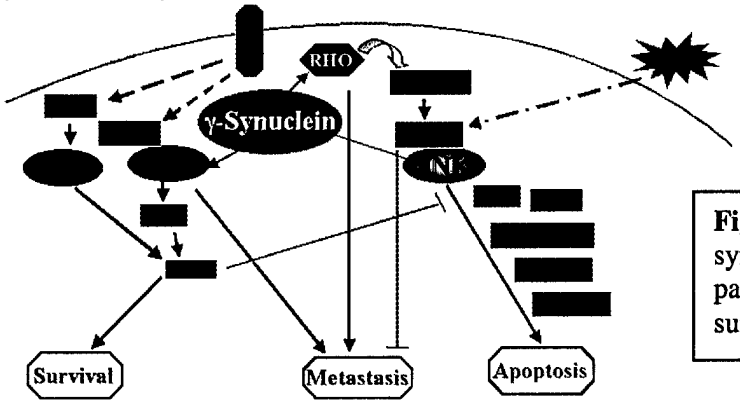


Figure 13. Diagram illustrating the effects of γ-synuclein on relevant signaling transduction pathways and their effects on metastasis, cell survival and apoptosis.

B-v-Polymorphisms of Human UGT1A6 and UGT1A9 Genes and Functional Differences of the Variant Gene Products.

Trainee: **Jeffrey Zalatoris, Ph.D.**
Mentor: **Rebecca Raftogianis, Ph.D.**

Introduction

Pharmacogenetic studies test the hypothesis that genetic variability of drug metabolizing enzymes is a contributing factor to variation in responses to drugs. UDP-glucuronosyltransferase (UGTs) enzymes are phase II drug metabolizing enzymes that detoxify endogenous and exogenous molecules, allowing for more rapid elimination of the conjugates from the body. Selective estrogen receptor modulators (SERMs) can be biologically inactivated by glucuronidation, and reports suggest that glucuronidation is a method of tamoxifen and raloxifene detoxification (70,71). I have hypothesized that genetic variation in the phenol glucuronidating enzymes UGT1A6 and UGT1A9 may predispose individuals to altered response to antiestrogens or increased risk of estrogen-dependent breast cancer. I have identified that UGT1A6 and UGT1A9 can, in fact, glucuronidate 4-hydroxytamoxifen, and I have identified that four UGT isoforms (1A1, 1A7, 1A8, and 1A9) can glucuronidate raloxifene. I have identified four UGT1A6 alleles arising from three common single-nucleotide polymorphisms (SNPs) that encode amino acid changes. I have genotyped healthy, ethnically defined populations for these common genetic polymorphisms of UGT1A6. I have correlated UGT1A6 genotype with the level of UGT biochemical activity in a population of human liver samples. Subsequently, I will biochemically characterize those variants using recombinant wild-type and variant UGT1A6 enzymes. Also, I have identified genetic polymorphisms in proximal, non-coding sequences near the human UGT1A9 gene. Finally, I will analyze the functional significance of UGT1A6 polymorphisms and UGT1A9 in a cell model system to determine the differential effects of UGT allozymes on the proliferation of the estrogen-dependent cells in the presence of estrogens and antiestrogens. These studies are designed to test the hypothesis that common genetic polymorphisms in UGT1A6 and UGT1A9 result in functional variability at the molecular level. In addition, I hypothesize that the molecular phenotypic variability may have clinical significance, and these genes may be important biological markers for human response to estrogens and antiestrogens.

Body

a-Glucuronide inactivation of 4-hydroxytamoxifen and raloxifene.

Commercially available UGT-containing microsomes were analyzed for the capacity to glucuronidate the selective estrogen receptor modulators (SERMs) 4- hydroxytamoxifen and raloxifene using a radiochemical assay designed to separate glucuronidated products from the starting substrates by thin-layer chromatography. The enzymes UGT1A6 and UGT1A9 could glucuronidate 4-hydroxytamoxifen (Figure 1), and the enzymes UGT1A1, UGT1A7, UGT1A8, and UGT1A9 could glucuronidate raloxifene (Figure 2). The glucuronidation of raloxifene by UGT1A1 appeared to only be able to conjugate one of the two phenolic moieties, while the other three UGT1A isoforms can glucuronidate both of the phenolic positions. Enzymes UGT1A3, UGT1A4, UGT1A10, UGT2B7, and UGT2B15 did not yield detectable products of either SERM.

b-Identification of UGT1A6 genetic variants.

In a population of 35 healthy Caucasians, complete sequencing of the coding region of the UGT1A6 first exon was conducted to identify genetic variants. The first exon was solely examined because this exon,

which contains more than half of the coding region for UGT1A6, is unique to UGT1A6, whereas the second through fifth exons of all human UGT1A genes are shared, and therefore are not specific for UGT1A6 enzyme variation. Single nucleotide polymorphisms (SNPs) were detected at UGT1A6 nucleotide positions 19, 315, 541, and 552, and the variants at positions 19, 541 and 552 are non-synonymous, resulting in the following amino acid changes to the enzyme: S7A, T181A, and R184S (Table 1). Within this population, these SNPs were found in four combinations as specific genotypes (Table 1). A PCR-RFLP genotyping assay was developed to rapidly characterize the UGT1A6 genotype in larger populations. Among 115 healthy Caucasians, the four different UGT1A6 alleles were identified in 62%, 27%, 5% and 5% of this population, and the population genotype data conformed to Hardy-Weinberg Equilibrium (Table 2) (72). Among 74 healthy African-American blood donors, the four UGT1A6 alleles were detected in 61%, 24%, 10%, and 5% of the population, and these data also conformed to Hardy-Weinberg Equilibrium.

c-Correlation of UGT1A6 genotype and function in liver tissues.

To test the initial hypothesis that genotype variation leads to phenotypic differences of UGT1A6, archived human liver tissue samples collected from surgical liver resections performed at the Fox Chase Cancer Center were obtained from the Fox Chase Cancer Center Tumor Bank and studied with IRB approval. From tissue homogenates, DNA was isolated to genotype the UGT1A6 gene, and microsomes containing UGT1A6 protein were isolated for functional characterization. These samples included 61 tumor tissues and 13 adjacent normal tissues. All tissues were genotyped for UGT1A6 and stratified as "normal" or "tumor" (Table 3). Distribution of alleles within the tumor population also adhered to Hardy-Weinberg Equilibrium (72).

Functional assays were developed to study the glucuronidation rate of UGT1A6 toward the highly specific substrate α-naphthol and the modestly specific substrate p-nitrophenol. The assays were developed to detect the spectral absorbance change upon glucuronide conjugation of the substrates. The glucuronidation rates for the two substrates were measured for all isolated microsomes containing UGT1A6. Table 4 and Figure 3 show the UGT1A6 glucuronidation rates as the mean value +/- error for the different genotyped tissues. ANOVA followed by Tukey's test were used to identify significant differences in the mean rate of glucuronidation. A significant difference in glucuronidation rates for both substrates was found for the UGT1A6*2/*2 genotyped microsomes than for the UGT1A6*1/*1, *1/*2, and *1/*3 genotyped microsomes ($p < 0.05$, $p < 0.01$, $p < 0.05$, respectively).

d-Correlation of UGT1A6 genotypes with level of expressed protein.

Using the same microsomal preparations isolated from human liver tissues, Western blots were performed on 50 μg total protein. Membranes were probed with an anti-UGT1A6 antibody (1:5000, Gentest) and an HRP-conjugated secondary antibody. Densitometry of the scanned film images was used to quantify the data. The results clearly indicated variations in the level of protein, and there was a positive correlation between the level of immunoreactive protein and the glucuronidation activity toward p-nitrophenol for tissues of *1/*1 ($n = 16$) and *1/*2 ($n = 28$) genotypes ($r^2 = 0.510$, $p < 0.01$ and $r^2 = 0.571$, $p < 0.001$) (Figure 4). While the level of immunoreactive protein varied by 85-fold, average values for tissues expressing the different genotypes were not significantly different. These differences could not completely account for the differences in glucuronidating activity by genotype, though the level of expressed UGT1A6 protein may have some role in the rate of glucuronidating activity.

e-Genetic polymorphisms detected near UGT1A9 first exon.

Direct DNA sequencing of the UGT1A9 first exon from 48 individuals was performed ranging from 273 nucleotides 5' of the start codon to 337 nucleotides into the first intron. Three SNPs were identified in the intron sequence at nucleotides 152, 219, and 313 from the exon/intron boundary. One variable T repeat (T_{9-10}) was identified in a putative TATA element (beginning at –118 nucleotides 5' of the start codon)(4), and an uncommon SNP (G/A) was identified at nucleotide –87, one of two putative mRNA cap sites (73). No coding region polymorphisms were detected in this screen. Comparing the various polymorphisms together, there are at least 8 different alleles with no polymorphisms found in the coding region (Table 5). The nucleotide variations to the proximal UGT1A9 first exon may have a role in genetic transcription or mRNA stability, which may be addressed in future studies. Even in the absence of functionally significant genetic variations to UGT1A9, the function of UGT1A9 may need to be more thoroughly examined not only because it is the only UGT identified that glucuronidates both 4-hydroxytamoxifen and raloxifene (Figures 1 and 2), but UGT1A9 has also been identified in human breast tissue (74), making it a candidate for drug metabolism at breast tissue as well as in other metabolic tissues.

f-Recombinant enzyme expression.

Recombinant proteins will be examined to further test the hypothesis that the genotypes result in functionally different phenotypes for UGT1A6. The pBlueBac/UGT1A6 vector construct, containing the UGT1A6*1 allele, was kindly provided by Dr. R. Tukey (University of California – San Diego). The gene was sub-cloned into a histidine-tagged baculoviral expression vector, pBlueBacHis2A. Site-directed mutagenesis was performed on the gene to generate the other three haplotypes of the UGT1A6 gene in the same vector, and these UGT1A6 constructs contained no spurious mutations as detected by complete DNA sequencing. Repeated attempts at overexpression of the UGT1A6 variants in insect cells were unsuccessful. Each of the four UGT1A6 variants was sub-cloned into the vector pcDNA3.1+zeo. For each of the four cDNA alleles of UGT1A6, genes have been stably transfected with the pcDNA3.1+zeo/UGT1A6 variant constructs into HEK293 mammalian cells, which lack endogenous UGTs. Specific activities of these expressed allozymes toward prototypic UGT1A6 substrates α-naphthol and p-nitrophenol as well as OHT will be characterized.

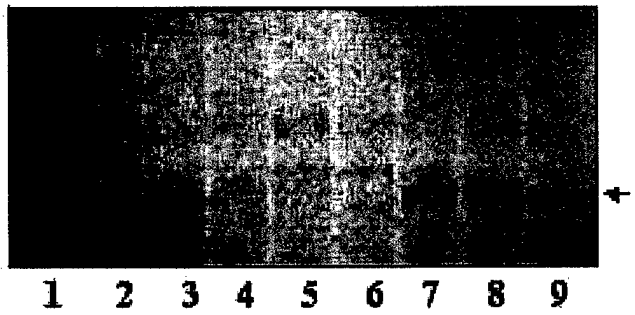


Figure 1. Glucuronidation of 4-hydroxytamoxifen (OHT) was measured by detecting the 14-C labeled reaction product 4-hydroxytamoxifen glucuronide (OHT-glc) from thin-layer chromatography on a silica gel plate. Images were detected by phosphorimager. Microsomes containing UGT1A6 and UGT1A9 glucuronidated OHT. Arrow indicates where bands of OHT-glucuronide have migrated on the plate. Lanes are: 1. UGT1A6 control (no OHT), 2-4. UGT1A6, 5-6. UGT1A9 control (no OHT), and 7-9. UGT1A9.

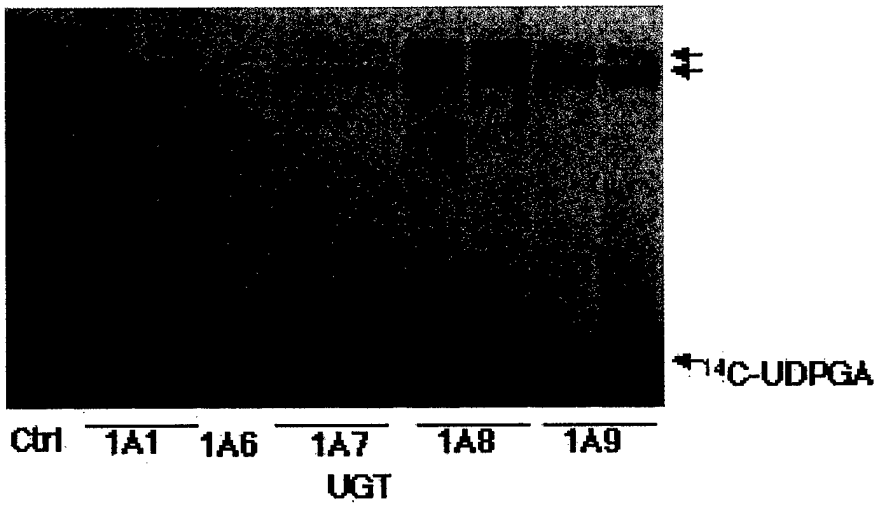


Figure 2. Glucuronidation of raloxifene (RAL) was measured by detecting the 14-C labeled reaction products from thin-layer chromatography. Upper bands represent the migration of the 14-C raloxifene-glucuronide (a single band for UGT1A1, and two bands for UGT1A7, 1A8, and 1A9). The lower bands represent the unincorporated cosubstrate 14-C UDPGA. Microsomes containing UGT1A1, UGT1A7, UGT1A8 and UGT1A9 glucuronidated RAL. No products were detected with microsomes containing UGT1A3, UGT1A4, UGT1A6, UGT1A10, UGT2B7, and UGT2B15 (not shown).

Table 1. **Common nucleotide and amino acid variations of the UGT1A6 gene**

	Nucleotide (Amino Acid)				Allele Frequencies (Number)	
Allele	19 (7)	315 (105)	541 (181)	552 (184)	Caucasian	African-American
*1	T (Ser)	A (Leu)	A (Thr)	A (Arg)	0.617 (142)	0.608 (90)
*2	G (Ala)	G (Leu)	G (Ala)	C (Ser)	0.274 (63)	0.243 (36)
*3	G (Ala)	A (Leu)	A (Thr)	A (Arg)	0.057 (13)	0.10 (15)
*4	G (Ala)	G (Leu)	A (Thr)	C (Ser)	0.052 (12)	0.047 (7)

Allele frequencies were determined by either DNA sequencing or application of the PCR-RFLP assay in a total of 115 healthy Caucasian and 74 healthy African-American subjects.

Table 2. UGT1A6 Genotype Frequencies in Healthy Caucasian Population (n= 115) Follow Hardy-Weinberg Equilibrium

Genotype	Observed		Predicted	
	#	Frequency	#	Frequency
*1/*1	46	0.400	44	0.380
*1/*2	37	0.320	39	0.340
*1/*3	6	0.052	8	0.070
*1/*4	7	0.061	7	0.064
*2/*2	11	0.096	9	0.075
*2/*3	3	0.026	4	0.031
*2/*4	1	0.009	3	0.029
*3/*4	4	0.035	1	0.006

Allele frequencies shown in Table 1 were used to calculate predicted values for Hardy-Weinberg Equilibrium. The Hardy-Weinberg Distribution program HWDIAG β1.0 was used to calculate the probability that these data fit the Hardy-Weinberg Equilibrium (3). The threshold for significant variance from Hardy-Weinberg Equilibrium was chosen as 0.05, and as the α* value (probability) approaches 1, the probability becomes greater that these data adhere to Hardy-Weinberg Equilibrium. These data had a α* value of 0.08, suggesting that the genotype frequencies of this population were distributed in accordance with Hardy-Weinberg Equilibrium.

Table 3. UGT1A6 Genotype Frequencies for Liver Tumors

Genotype	Observed (n=62)		Predicted	
	#	Frequency	#	Frequency
*1/*1	19	0.306	23	0.376
*1/*2	35	0.564	26	0.425
*1/*3	2	0.032	2	0.039
*1/*4	1	0.016	1	0.010
*2/*2	3	0.048	7	0.120
*2/*3	2	0.032	1	0.022
*2/*4	0	0.000	0	0.006
*3/*4	0	0.000	0	0.000

Allele frequencies were used to calculate the predicted values for Hardy-Weinberg Equilibrium. Using HWDIAG β1.0 (3), these data had a α* value (probability) of 0.11, greater than the threshold for significant variance from Hardy-Weinberg Equilibrium (0.05) thus implying that the population distribution of these genotypes was in equilibrium.

Table 4. Glucuronidation Rates of α-Naphthol and p-Nitrophenol by UGT1A6 Genotype

UGT1A6 Genotype	(#)	Rate of α-Naphthol Glucuronidation	Error (+/-)	Rate of p-Nitrophenol Glucuronidation	Error (+/-)
*1/*1	(22)	14.1	2.52	4.58	0.88
*1/*2	(42)	9.30	1.04	2.38	0.40
*1/*3	(2)	1.18	1.18	0.39	0.31
*1/*4	(1)	13.1	N/A	0	N/A
*2/*2	(4)	28.0	1.25	9.60	1.04
*2/*3	(3)	17.4	2.92	8.85	0.68

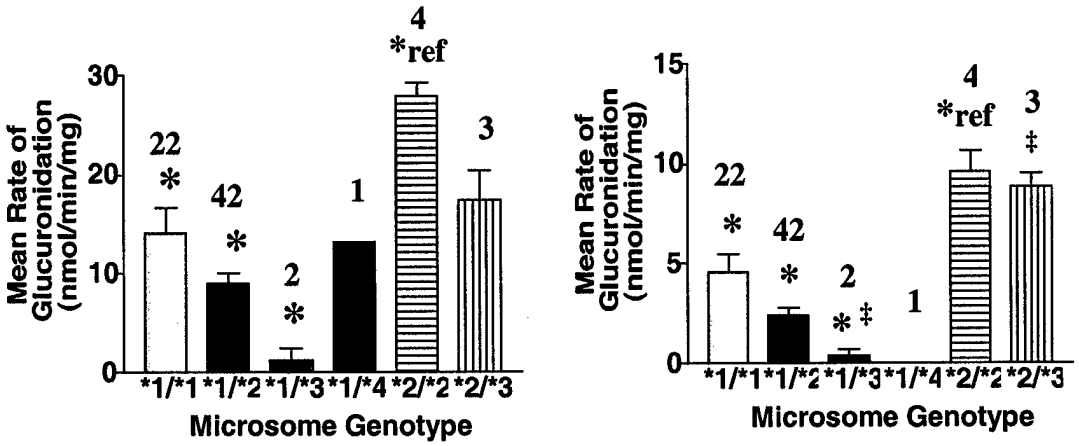


*Figure 3. UGT1A6*2 homozygous liver microsomes glucuronidate simple phenols at greater rates than samples that were homozygous for UGT1A6*1 or heterozygous for *1/*2 or *1/*3. Mean glucuronidation rates of α-naphthol (A) and p-nitrophenol (B) were determined by spectrophotometric analyses. The number of liver microsomes associated with each genotype are listed above each error bar. ANOVA followed by Tukey's comparison test was performed. In both A and B, each of the asterisked bars represented significantly different mean glucuronidation rates for these genotyped samples from the mean rate of UGT1A6*2/*2 (*ref): *1/*1, *1/*3, and *1/*2 ($p < 0.05$, $p < 0.05$, $p < 0.01$, respectively). In B, the mean glucuronidation rate of p-nitrophenol by UGT1A6*1/*3 was significantly different from that of the UGT1A6*2/*3 ($p < 0.05$)(‡).*

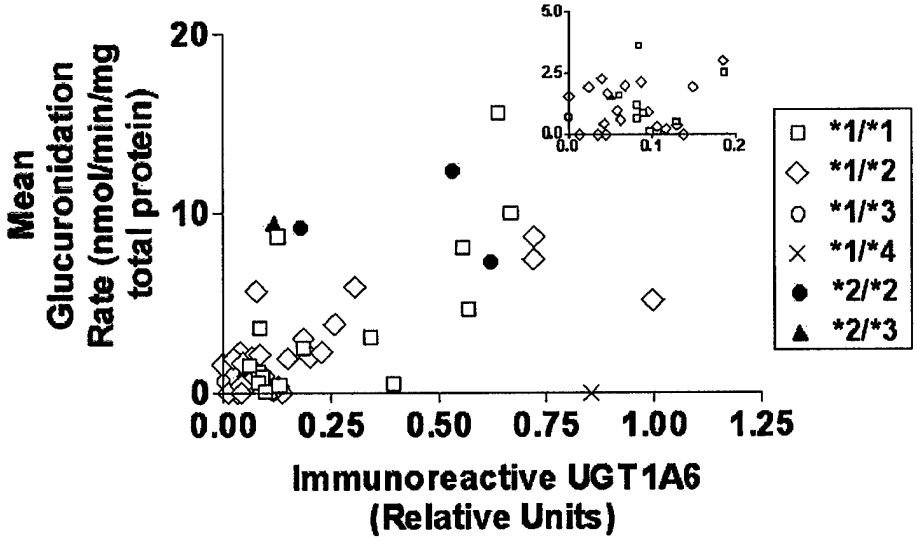


Figure 4. Mean glucuronidation rate correlates with level of expressed protein from individual human liver tumor tissues. 50 μg of microsomal protein were separated by gel electrophoresis. Immunoblots were probed with the α-UGT1A6 primary antibody (Gentest). For each gel, three concentrations of recombinant UGT1A6 standards (1 relative unit = 1 μg) were also evaluated to generate a standard curve for quantification of immunoreactive UGT1A6. UGT1A6*1/*1 ($n = 16$, $r^2 = 0.510$) and UGT1A6*1/*2 ($n = 28$, $r^2 = 0.571$) showed positive correlation between level of expressed protein and glucuronidation of p-nitrophenol. Inset in figure is an expansion of the area nearest the origin.

Table 5. Genetic Polymorphisms Identified NearUGT1A9 First Exon

	Polymorphisms					
Genotypes	-118 dT	-87	I152	I219	I313	NUMBER
*1/*1	10	G	G	A	C	8
*2/*2	9	G	A	T	A	6
*3/*3	9	G	G	T	A	3
*2/*3	9	G	A/G	T	A	6
*1/*3	9/10	G	G	A/T	A/C	12
*1/*2	9/10	G	A/G	A/T	A/C	4
*1/*4	10	G	G	A/T	C	3
*3/*5	9	G	G	A/T	A/C	2
*2/*6	9/10	G	A	A/T	A/C	1
*1/*7	10	G	G	A/T	A/C	1
*2/*8	9	A/G	A/G	T	A	1
*1/*8	9/10	A/G	G	A/T	A/C	1

Discussion

UGT1A6 and UGT1A9 have been identified to glucuronidate 4-hydroxytamoxifen, and UGT1A1, 1A7, 1A8 and 1A9 glucuronidate raloxifene. Four single nucleotide polymorphisms in human UGT1A6 have been shown to exist in four combinations (Table 1). These four alleles are present in 62%, 27%, 5%, and 5% of the healthy Caucasian population analyzed, and 61%, 24%, 10% and 5% of the healthy African-American population. Archived liver tissues were obtained from persons who had undergone surgical resection for liver cancer. These samples were genotyped and rates of glucuronidation for two substrates of UGT1A6 were measured. ANOVA indicated significant variations in the glucuronidation rates for the tissues with the UGT1A6*2/*2 genotype compared to those tissues with the *1/*1, *1/*2 and *1/*3 genotypes. Notably, a 25-fold difference in glucuronidation rates was observed between liver tissues expressing UGT1A6*1/*3 versus those only expressing UGT1A6*2. The initial hypothesis that genotype variation would result in functional variants was supported by this experiment. These analyses will be verified by studying the glucuronidation rates of recombinantly expressed UGT1A6 allozymes. Stable transfection of the UGT1A6 variants has been successful in expressing functional products in mammalian HEK293 cells. One possibility exists that the differences in protein functional turnover were directly related to the level of expressed UGT1A6 in the liver tissues. Therefore, the level of immunoreactive UGT1A6 enzyme was measured in the liver tissue microsomes. Tissues expressing the more common genotypes exhibited a positive correlation between the glucuronidation rate and level of protein. However, the level of UGT1A6*2/*2 tissues did not correlate with activity, suggesting another mechanism for the difference in glucuronidation rate (e.g. kinetic differences). A thorough screening for genetic variants of the UGT1A9 gene identified non-coding region polymorphisms. Such variants may alter gene transcription and stability of mRNA. The examination of the variant genes may provide critical insight about which of the polymorphic amino acids are critical for phenotypic differences (UGT1A6) and which of the nucleotide changes might alter phenotypes (UGT1A9) *in vivo*.

C- KEY RESEARCH ACCOMPLISHMENTS.

C-i- ***Study of the functional role of chromosomes 11, 13, 14 and 17 in the process of immortalization, transformation and tumorigenesis of human breast epithelial cells, by Hasan Lareef, MD.***

a-The chemical carcinogen benz (a) pyrene induces transformation of MCFl0F cells as evidenced by anchorage independence; loss of ductulogenic capacity in collagen and loss of activation of Fas mediated apoptosis. These transformation phenotypes are associated with LOH in chromosome 17p13.1-13.2 regions (Dl7S796).

b-Transfer of chromosome 17 p13.1-13.2 (D17S796) region reverts the transformation phenotypes as well as Fas mediated apoptosis. We postulate that the chromosome 17 (pl3.1-13.2) (Locus D17S796) region may contain gene/s responsible for maintaining ductulogenic capacity in collagen, colony formation in agar-methocel and controlling programmed cell death through FAS receptor/ligand complex.

c-Ras oncogene in BP transformed cells(BP1-Tras) turn them in highly tumorigenic in SCID mice. This phenotype is associated with LOH in several loci of chromosome 13.

d-Transfer of chromosome 13 to BP1-Tras cells abrogates the tumorigenesis in SCID mice.

e-Transfer of chromosome 14 to BP1 T ras cells does not abrogate the tumorigenesis in SCID mice.

C-ii. Lymphedema Prevention in Breast Cancer Survivors (Project 1) and Knowledge About Genetic Risk Assessment and Interest in Genetic Risk Assessment for Hereditary Breast/Ovarian Cancer Among Inner City Women (Project 2), by: Kerry Sherman, Ph.D.

a-Obtained funding through a DOD Breast Cancer Idea Award (Co P.I) to study cognitive-affective predictors of the uptake of, and sustained adherence to, lymphedema symptom minimization practices in breast cancer survivors.

b-Commenced a pilot study (N = 4) related to lymphedema prevention among early stage breast cancer survivors to determine the feasibility of the study questionnaire and to determine the most appropriate and effective recruitment approach.

c-Data collection for the study entitled "Knowledge about genetic risk assessment and interest in genetic risk assessment for hereditary breast/ovarian cancer among inner city women" is ongoing (N = 78). Interim analyses have been conducted on these data. Specifically, level of genetics knowledge was strikingly low, yet interest to pursue breast cancer risk assessment counseling/genetic testing was high. Interest in risk assessment was associated with increased perceived risk for ovarian cancer, increased perceived control over ovarian cancer, and enhanced social support. In addition, greater social support was associated with increased interest in undergoing genetic testing.

C-iii. Roles of γ-synuclein in breast cancer progression and metastasis, by: Zhong-Zong Pan, Ph.D.

a-Overexpression of γ-synuclein leads to constitutive activation of ERK and Rho/Rac/Cdc42 and down-regulation of JNK activation in response to stress signals or chemotherapy drugs.

b-Overexpression of γ-synuclein induces stress fiber formation and enhances cell migration. Both the basal level and the enhanced cell migration require the activities of both the ERK and Rho/Rac/Cdc42 kinases.

c-Overexpression of γ-synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway.

c-iv. Polymorphisms of Human UGT1A6 and UGT1A9 Genes and Functional Differences of the Variant Gene Products, by: Jeffrey Zalatoris, Ph.D.

a-Identification of specific UGT enzymes that can catalyze the glucuronidation and, hence, inactivation of 4-hydroxytamoxifen (UGT1A6 and UGT1A9) and raloxifene (UGT1A1, UGT1A7, UGT1A8, and UGT1A9).

b human UGT1A6 haplotypes were identified as variant combinations of 4 single nucleotide polymorphisms.

c-Genotype assay (PCR-RFLP) was developed for rapid screening of UGT1A6 polymorphisms.

d-Novel α-naphthol and p-nitrophenol glucuronidation assays were developed and applied to analyze rates of glucuronidation in a bank of human liver tissue.

e-Discovery that UGT1A6 glucuronidation was significantly greater for tissues with UGT1A6*2/*2 genotype than tissues genotyped as *1/*1, *1/*2, and *1/*3 ($p < 0.05$, $p < 0.01$, $p < 0.05$).

f-Discovery that level of protein positively correlates to glucuronidation rate for the tissues genotyped UGT1A6*1/*1 and UGT1A6*1/*2, but not for UGT1A6*2/*2.

g-HEK293 cells were stably transfected with the four haplotypes of UGT1A6.

D-REPORTABLE OUTCOMES (See Appendix, Exhibit B):

D-i-Study of the functional role of chromosomes 11, 13, 14 and 17 in the process of immortalization, transformation and tumorigenesis of human breast epithelial cells, by: Hasan, M. Lareef, M.D.

Abstracts

1-Mello, M.L.S., B.de Campos Vidal, Lareef, M.H., J.Russo. Changes in chromatin texture in transformed cells as assessed by molecular biology assays and image analysis. 14th international Congress of Cytology May 27-31 Amsterdam-The Netherland.

2-Mohamed H. Lareef, Irma H. Russo, Fathima S Sheriff, Jorge Thomas, Andrew Cuthbert, and Jose Russo. Transfer of chromosome 13 abrogates the expression of immortalization, in vitro phenotypes and tumorigenesis in human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 43:5-6, 2002.

3-Shawn Straszewski, Joon Song, Mohamed Lareef, Bozena Hanczaruk, Marijke Kamsteeg, Jose Russo and Gil Mor. The regulation of FLIP in Human Breast Epithelial Cells. Journal of the society for Gynecologic investigation supplement. 49th annual meeting Los Angeles, CA. 2002

Publications

1-Mello, M.L.S., Lareef, M.H., Vidal, B.C. and Russo, J. RNA relocation at mitosis in benz (a) pyrene transformed human breast epithelial cells after microcell mediated transfer of chromosomes 11 and 17. Analytical Cellular Pathology, 23:137-141, 2001.

Manuscript in preparation

1-M.H. Lareef, J.Song Q. Tahin, I.H. Russo, G.Mor, D. Mihaila, C.Slater, A. Cuthbert, B. Balsara, J. Testa, D.Broccoli, J.V.Grobelny and J. Russo. Microcell Mediated chromosome 17 transfer to chemically transformed Human Breast Epithelial Cells induces Fas mediated apoptosis and reverts the transformation Phenotypes.

D-ii-Lymphedema Prevention in Breast Cancer Survivors (Project 1) and Knowledge About Genetic Risk Assessment and Interest in Genetic Risk Assessment for Hereditary Breast/Ovarian Cancer Among Inner City Women (Project 2), by: Kerry Sherman, Ph.D.

Abstracts

1-Sherman, K., Montrone, M., Tyler, J., Driscoll, G., & Miller, S.M. (2002) The 23rd International Conference of the Stress and Anxiety Research Society. Paper on: Coping strategies, monitoring attentional style and infertility-related distress among couples undergoing in-vitro fertilization. Melbourne, Australia, July, 2002.

2-Sherman, K.A., Miller, S.M., Rodoletz, M., Driscoll, J., Daly, M., Godwin, A., & Babb, J. The 23rd Annual Meeting of the Society of Behavioral Medicine. Paper on: The role of monitoring and anticipated *BRCA1/2* carrier status on family communications intentions and plans among women with a hereditary pattern. Washington, DC, April, 2002.

3-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., & Babb, J. (2002). The 23rd Annual Meeting of the Society of Behavioral Medicine. Poster on: Enhancing Adaptation to Prostate Cancer Risk Feedback and Screening Adherence. Washington, DC, April, 2002.

4-Sherman, K., Montrone, M., Miller, S.M., Tyler, J., M.D., & Driscoll, G. (2002) The 23rd Annual Meeting of the Society of Behavioral Medicine. Poster on: Psychosocial predictors of pregnancy outcome among couples undergoing In-Vitro Fertilization. Washington, DC, April, 2002.

5-Miller, S.M., Driscoll, J., Rodoletz, M., Sherman, K., Buzaglo, J., Daly, M., Godwin, K., & Babb, J. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: Attentional style and adjustment to participation in genetic testing for inherited breast and ovarian cancer risk. Bethesda, MD, March, 2002.

6-Miller, S.M., Sherman, K., Rodoletz, M., Buzaglo, J., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: The role of monitoring and anticipated BRCA1/2 carrier status on family communication among women with a hereditary pattern. Bethesda, MD, March, 2002.

7-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., & Bruner, D. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: Prostate Cancer Risk Assessment: Enhancing Participation and Adaptation. Bethesda, MD, March, 2002.

8-Miller, S.M., Sherman, K., Rodoletz, M., Buzaglo, J., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (2002) The 60th Annual Meeting of the American Psychosomatic Society. *Citation poster* on: The role of monitoring and anticipated BRCA1/2 carrier status on family communication intentions and plans among women with a hereditary pattern for breast/ovarian cancer. Barcelona, Spain, March, 2002.

9-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., & Babb, J. (2002). The 60th Annual Meeting of the American Psychosomatic Society. Poster on: Enhancing Adaptation to and Participation in Prostate Cancer Risk Programs. Barcelona, Spain, March.

10-Sherman, K., Montrone, M., Tyler, J., Driscoll, G., & Miller, S.M. (2002). The 60th Annual Meeting of the American Psychosomatic Society. *Press release Poster* on: Coping strategies and pregnancy outcome among couples undergoing In-Vitro Fertilization. Barcelona, Spain, March, 2002.

11-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., Bruner, D., & Knowles, J.C. (2002). The 2nd Annual Meeting of the American Academy of Health Behavior. Poster on: Enhancing Participation in and Adaptation to Prostate Cancer Risk Assessment Programs and Screening Adherence. Napa Valley, CA, March, 2002.

12-Sherman, K.A., Montrone, M., Tyler, J., Driscoll, G., Miller, S.M., & Knowles, J. (2002). The 2nd Annual Meeting of the American Academy of Health Behavior. Poster on: Psychosocial predictors of pregnancy outcome among couples undergoing In-Vitro Fertilization. Napa Valley, CA, March, 2002.

Publications

1-Miller, S.M., Sherman, K.A., Buzaglo, J.S., & Rodoletz, M. (2001). Monitoring-Blunting behavioral signatures in coping with health threats: The example of cancer. Psicologia della Salute, 3, 37-48.

2-Sherman, K.A., Miller, S.M., & Sheinfeld-Gorin, S. (Under review). Psychosocial determinants of participation in risk counselling programs and breast cancer screening regimens among African American women. Breast Cancer in African American Women Monographs, Susan G. Komen Foundation and the African American National Advisory Committee.

Manuscripts in preparation

1-Diefenbach, M.A., Sherman, K.A , & Croyle, R. (In preparation). ASPO 2002 Roundtable Session 3: What is the Role of Risk Perceptions in Cancer Screening Adherence? Cancer, Epidemiology, Biomarkers and Prevention.

2-Miller, S.M., Sherman, K., Rodoletz, M., Buzaglo, J., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (In preparation). The role of monitoring and anticipated BRCA1/2 carrier status on family communication among women with a hereditary pattern.

3-Miller, S.M. & Sherman, K.A. (In Preparation). Cancer Screening chapter, Encyclopedia of Health and Behavior (Norman Anderson, Ed.)

Grant funding obtained

Department of Defense Breast Cancer Idea award (USA) – Co-P.I. – "Cognitive-Affective Predictors of the Uptake of, and Sustained Adherence to, Lymphedema Symptom Minimization Practices in Breast Cancer Survivors" - $448,000 – 40% effort.

Grants pending

New Jersey Department of Health and Senior Services – Co P.I. - Evaluation of Barriers to Follow-Up Diagnostic and Treatment Adherence Among Underserved Women in the New Jersey Breast Cancer Education and Early Detection (CEED) program - $100 000 - 10% effort.

D-iii. Roles of γ-synuclein in breast cancer progression and metastasis, by: Zhong-Zong Pan, Ph.D.

Abstracts

1-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y, and A.K. Godwin (2002). Gamma-synuclein is over-expressed in breast and ovarian cancers and promotes tumor cell survival by inhibiting stress-induced apoptosis. AACR 93rd Annual Meeting, San Francisco, April 6-10, 2002.

2-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y, and A.K. Godwin (2001). Gamma-synuclein, a candidate oncogene, activates Rac and ERK and contributes to the metastatic spread of breast and ovarian cancer. 51st Annual Meeting of the American Society of Human Genetics, San Diego, Oct. 12-16, 2001.

3-Frolov, A., Pan, Z.Z., Broccoli, D., Giaisson, B.I., Verderveer, L., Auersperg. N., Lynch, H., Daly, M., Hamilton, T. and A.K. Godwin (2001). Identification of ovarian cancer-associated genes using a HOSE cell transformation model. NCI (National Cancer Institute) 9th SPORE (Specialized Program of Research Excellence) Investigators' Workshop, Washington, DC, July 15-17, 2001.

Publications

1-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y., Godwin, A.K. Gamma-synuclein promotes cancer cell survival and inhibits stress- and chemotherapeutic drug-induced apoptosis by modulating MAPK pathways. (Revised manuscript, JBC2002).

2-Frolov, A., Arnoletti, J.P., Pan, Z.Z., von Mehren, M., Eisenberg, B. and A.K. Godwin. *Sprouty* 4A, a novel genetic marker of response to imatinib mesylate in gastrointestinal stromal tumors. (Submitted, Nature Medicine 2002).

3-Prowse, A.H., Vanderveer, L., Milling, S.W.F., Pan, Z.Z., Dunbrack, R.L., Xu, X.X., and A.K Godwin (2002). OVCA2 is down-regulated and degraded during retinoid-induced apoptosis. Int. J. Cancer 99:185-192.

D-iv. Polymorphisms of Human UGT1A6 and UGT1A9 Genes and Functional Differences of the Variant Gene Products, by:Jeffrey Zalatoris, Ph.D.

Abstracts

1-Presentation at the 2001 Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, Orlando, FL, March, 2001.

2-"UGT1A6 Genetic Polymorphisms: Identification and Genotype/ Phenotype Analysis from Human Liver Tissues." J.J. Zalatoris, PhD* and R.B. Raftogianis, PhD, Dept. Pharmacology, Fox Chase Cancer Center, Philadelphia, PA.

Manuscript in preparation "

1-Association Between Human UGT1A6 Haplotype and Level of Enzyme Activity in Human Liver Tissue." J.J. Zalatoris and R.B. Raftogianis.

E-CONCLUSIONS:

E-i.- Study of the functional role of chromosomes 11, 13, 14 and 17 in the process of immortalization, transformation and tumorigenesis of human breast epithelial cells, by Hasan, M. Lareef, MD

Our work demonstrates that the chemical carcinogen benz (a) pyrene induces transformation of MCFl0F cells as evidenced by anchorage independence; loss of ductulogenic capacity in collagen and loss of activation of Fas mediated apoptosis. These transformation phenotypes are associated with LOH in chromosome 17p13.1-13.2 regions (Dl7S796). Transfer of chromosome 17 p13.1-13.2 (D17S796) region reverts the transformation phenotypes as well as Fas mediated apoptosis. We postulate that the chromosome 17 (pl3.1-13.2) (Locus D17S796) region may contain gene/s responsible for maintaining ductulogenic capacity in collagen, colony formation in agar-methocel and controlling programmed cell death through FAS receptor/ligand complex. Ras oncogene turns the BP-transformed cells in a highly tumorigenic cell in the SCID mice. The tumorigenic phenotype is associated with alterations at different loci of chromosome 13. Transfer of this chromosome but not chromosome 14 is able to abrogate the tumorigenic phenotype. All together our data are providing a powerful experimental system to dissect the different genes involved in the initiation and progression of breast cancer.

E-ii.- Lymphedema Prevention in Breast Cancer Survivors, by Kerry Sherman, Ph.D

a-Obtained funding through a DOD Breast Cancer Idea Award (Co P.I) to study cognitive-affective predictors of the uptake of, and sustained adherence to, lymphedema symptom minimization practices in breast cancer survivors.

b-Commenced a pilot study (N = 4) related to lymphedema prevention among early stage breast cancer survivors to determine the feasibility of the study questionnaire and to determine the most appropriate and effective recruitment approach.

c-Data collection for the study entitled "Knowledge about genetic risk assessment and interest in genetic risk assessment for hereditary breast/ovarian cancer among inner city women" is ongoing (N = 78). Interim analyses have been conducted on these data. Specifically, level of genetics knowledge was strikingly low, yet interest to pursue breast cancer risk assessment counseling/genetic testing was high. Interest in risk assessment was associated with increased perceived risk for ovarian cancer, increased

perceived control over ovarian cancer, and enhanced social support. In addition, greater social support was associated with increased interest in undergoing genetic testing.

E.-iii.-. Roles of γ-synuclein in breast cancer progression and metastasis, by Zhong-Zong Pan, Ph.D.

In these studies, we found that γ-Synuclein can interact with two major MAPKs, i.e., ERK and JNK1. Over-expression of γ-synuclein may lead to enhanced activity of ERK and down-regulation of JNK activation in response to stress and chemotherapy drugs. Rho/Rac/Cdc42 pathway is also activated in cells over-expressing γ-synuclein. Activation of both the Rho/Rac/Cdc42 and ERK pathways are required for the enhanced cell migration in γ-synuclein over-expressing cells. Over-expression of γ-synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway. Taken together, these data indicate that γ-synuclein may promote tumorigenesis by enhancing cell motility through modulating Rho/Rac/Cdc42 and ERK pathways, and promoting cell survival and inhibiting apoptosis through modulating ERK cell survival and JNK-mitochondria-caspase9/3 apoptotic pathways. Since γ-synuclein is aberrantly expressed in the majority of late-stage breast and ovarian cancers but is not expressed in normal breast and ovarian epithelial cells, γ-synuclein may represent a very promising therapy target for these diseases.

E.-iv. Polymorphisms of Human UGT1A6 and UGT1A9 Genes and Functional Differences of the Variant Gene Products, by Jeffrey Zalatoris, Ph.D.

A principal focus of pharmacogenetics is the identification of genetic variants of drug metabolizing enzymes that result in different drug responses in humans. UGT glucuronidation of selective estrogen receptor modulators has led to the hypothesis that phenol glucuronidating enzymes are at least partially responsible for the bioinactivation of SERMs. Because SERMs are being prescribed as long-term preventative therapies against the development of breast cancer, the response of patients to these drugs will likely dictate the patients' adherence to the therapy regimen and the acceptance of any costs in terms of financial and health issues. Therefore the identification of genetic factors influencing responses to these therapies may aid physicians in their ability to prescribe the drugs effectively. This study is aimed at defining whether these putative SERM inactivating enzymes have genetic variations that result in predictable phenotypes at both the molecular level and in the patient.

Since UGT1A6 and UGT1A9 were shown to glucuronidate 4-hydroxytamoxifen, these genes were studied for potential genetic variants that might affect the phenotype of the gene products. UGT1A6 genotype has been determined in both healthy Caucasian and African-American populations and in a population of liver tumor tissues from patients with liver cancers. The study of liver tissue genotype and phenotype was performed because these tissues are rich in UGT1A6 and supplied enough material to compare glucuronidation rates among different genotyped samples. In the study of liver tissue glucuronidation of the highly specific UGT1A6 substrate α-naphthol and the more general UGT substrate p-nitrophenol, the liver microsomes homozygous for UGT1A6*2/*2 showed statistically greater rates of glucuronidation than the microsomes genotyped as *1/*1, *1/*2, and *1/*3. In fact, a 25-fold difference was detected between tissues expressing the UGT1A6*1/*3 allozymes and those that were homozygous for the UGT1A6*2. These data suggest that the *2 allozyme has a greater specific activity in liver than the *1 allozyme. Because of the rarity of the *3 and *4 genotypes, no homozygous liver tissues have been identified from this population. Therefore, to study the effect of specific activity variation due to the genotype, expression of recombinant proteins for all of these variants will need to be performed. To address the hypothesis that variant allozymes result in functional changes to the inactivation of estrogens and antiestrogens *in vivo*, a cell model study will be conducted to assess the proliferation of estrogen-dependent mammalian cells stably transfected with the variant UGT1A6 genes in the presence and

absence of SERMs. Differences in the stability of the UGT1A6 allozymes will also be studied using these cell lines. Finally, the identification of non-coding region UGT1A9 genetic polymorphisms may hypothetically lead to variant gene transcription or mRNA stability. Clinical variation of the efficacy of SERMs may be dependent on a variety of factors, and the current studies are intended to identify potential molecular bases for such interindividual differences.

F. REFERENCES

1. Lee EY-HP: Tumor suppressor genes and their alterations in breast cancer. Semin cancer Biol: 119-125, 1995.
2. Bieche I and Liereau R: Genetic alterations in breast cancer. Genes chromosomes cancer 14:227-251, 1995.
3. Coleman WB and Tsongalis GJ: Multiple mechanisms account for genomic instability and molecular mutation in neoplastic transformation. Clin Chem 41:644-657, 1995.
4. Loeb LA: Many mutations in cancer. Cancer surv 28:329-342, 1996.
5. Fournier REK and Ruddle FH: Microcell-mediated chromosome transfer of murine chromosome into mouse, Chinese hamster, and human somatic cells. Proc of Natl acad Sci USA 74:319-323.
6. Oshimura M: Lessons learned from studies on tumor suppression by microcell mediated chromosome transfer. EnvironHealth Perspect 93: 57-58, 1991.
7. Anderson MJ and stanbridge EJ: Tumor suppressor genes studied by cell hybridization and chromosome transfer. FASEB J 7: 826-833, 1993.
8. Hunt JD: Evaluation of phenotypic alteration by microcell mediated chromosome transfer. Anal Biochem 2338:107-116, 1996.
9. Tsukamoto K. Emi M. Nakamura Y. [Cytogenetic abnormalities, genetic alterations, and applications for genetic diagnosis in breast cancer].
10. [Review] [22 refs] [Japanese] Nippon Rinsho - Japanese Journal of Clinical Medicine. 54(12):3389-97, 1996 Dec.
11. Murakami YS, Brothman AR, Leach RJ, and White RL: Suppression of malignant phenotype in a human proatete cancer cell line by fragments of normal chromosomal region 17q. Cancer Res 55:3389-3394, 1995.
12. Robertson G, Coleman A and Lugo TG: A malignant melanoma tumor suppressor on human chromosome 11. Cancer Res 56:4487-4492, 1996.
13. Plummer SJ, Adams L, Simmons JA and Casely G: Localization of agrowth suppressor activity in MCF7 breast cancer cells to chromosme 17q24-25. Oncogene 14: 2339-2345, 1997.
14. Oshimura M, Shimizu M, and Kugoh H: Genetic regulation of telomerase in a multiple pathways model to cellular senescence. Human Cell 9:301-308, 1996.
15. Nibel N, Ichikawa T, Kawana Y, Kuramochi H, Kugoh H, Oshimura M, Hayatai I, Shimazaki J and Ito H. Mapping of metastasis suppressor gene/s for rat prostate cancer on the short arm of human chromosome 8 by irradiated microcell-mediated chromosome transfer.Genes chromosome Cancer 17: 260-268, 1995.
16. Ischikawa T, Nihei N, Kuramochi H, Kawana Y, Killary AM, Rinker-Scharffer CW, Barrett JC, Isaacs JT, Kugoh H, Oshimura M and Shimazaki J; Metastasis suppressor genes for prostate cancer. Prostate Suppl 6:31-35, 1996.
17. Soule HD, Maloney TM, Woleman SR, Peterson WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF and Brooks SC. Isolation and charectarization of a spontaneously immortalized human breast epithelial cell line, MCF10F. Cancer Res 50:6075-6086, 1990.
18. Calaf G and Russo J: Transformation of human breast epithelial cells by chemical carcinogen. Carcinogenesis 14:483-492, 1993.
19. Russo J, Barnabas N, Zhang PL, and Adesina K: Molecular basis of breast cell transformation. Radiat Oncol Invest #; 424-429, 1996.

20. Huang Y, Bove B, Wu Y, Russo IH, Tahin Q, Yang X, Zekri A and Russo J: Microsatellite instability during the immortalization and transformation of human breast epithelial cells *in vitro.* Mol Carcinog 24: 118- 127, 1999.
21. Wu YL, Barnabas N, Russo IH, Yang X and Russo J: Microsatellite instability and loss of heterozygosity in chromosomes 9 and 16 in human breast epithelial cells transformed by chemical carcinogens. Carcinogenesis 18: 1069-1074,1997.
22. Russo J, Barnabas N, Higgy N, Salicioni AM, Wu YL and Russo IH: Molecular basis of human breast epithelial cell transformation. In: Breast Cancer. Advances in Biology and Therapeutics.Calvo F,Crepin M and Magdelenat H (eds). John Libbey Eurotext, pp33-43, 1996.
23. Barnabas N, Moraes R, Calaf G, Estrada S and Russo J: Role of p53 in MCF-IOF cell immortalization and chemically-induced neoplastic transformation. Int J Oncol 7: 1289-1296,1995.
24. Zhang PL, Calaf G and Russo J: Allele loss and point mutation in codon 12 and 61 of c-Ha-ras oncogene in carcinogen transformed human breast epithelial cells. Mol Carcinog 9: 46-56,1994.
25. Zhang PL, Chai YL, Ho TH, Calaf G and Russo J: Activation of c-myc, c-neu, and int-2 oncogenes in the transformation of HBEC MCF-IOF treated with chemical carcinogens *in vitro.* Int J Oncol 6: 963 968, 1995.
26. Yang X., Tahin Q, Yun Fu Hu, Irma Russo, Benaifer R Balsara, Dana Mihaila, Carolyn Slater J. Carl Barrettt and J.Russo. Functional role of chromosome11 and 17 in the transformation of human breast epithelial cells in vitro. Int J. Oncology 15: 629-638,1999.
27. Song J. Sapi E. Brown W. Nilson J. Tartaro K. Kacinski BM. Craft J. Naftolin F. Mor G. Role of Fas and Fas ligand during mammary gland remodeling.
28. Ungefroren H. Voss M. Jansen M. Roeder C. Henne-Bruns D. Kremer B. Kalthoff H: Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas mediated apoptosis. Cancer Research. 58(8):1741-9, 1998 Apr 15.
29. Mor G. Kohen F. Garcia- Velasco J. Nilsen J. Brown W. Song J. Naftolin F. Regulation of Fas ligand expression in breast cancer cells by estrogen: Functional differences between estradiol and tamoxifen. Journal of steroid Biochemistry & Molecular Biology. 73(5):185-94, 2000 Jul-Aug.
30. Solary E, Dron N, Bettaieb A, Corcos L, Dimanche-Biotrel MT, Garrido C. Positive and negative regulationof apoptosis pathways by cytotoxic agents in hematological malignancies. Leukemia 2000 Oct;14(10):1833-49.
31. Russo J, Calaf G, Sohi N, Tahin Q, Zhang PL, Alvarado ME, Estrada S and Russo Ih: Critical steps in breast carcinogenesis. Ann NY Acad Sci 698:1-20, 1993.
32. Bond JA, Willie FS and Wynford-ThomasD: Escape from senescence in human diploid fibroblasts induced directly by mutant p53. Oncogenee 7:1885-1888, 1994.
33. Harley CB: Telomere loss: mitotic clock or genetic time bomb? MutatRes256: 271-282,1991.
34. Hopfer U, Jacobberger JW, Gruenert DC, Eckert RL, Jat RS and Whitsett JA: Immortalization of epithelial cells. Am J Physiol270: Cl-C11, 1996.
35. Blackburn EH: Telomerase. Annu Rev Biochem 61: 113-129, 1992.
36. Shay JW, Wright WE and Werbin H: Toward a molecular understanding of human breast cancer: a hypothesis. Breast Cancer Res Treat 25: 83-94, 1993.
37. Bacchetti S and Counter CM: Telomeres and telomerase in human cancer. Int J Oncol 7: 423-432, 1995.
38. Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S and Greder CW: Human telomerase RNA and telomerase activity in immortal cells lines and tumor tissues. Cancer Res 56: 645-650, 1996.
39. Soussi T, Legros Y, Lubin R, Ory K and Schlichtholz B: Multifactorial analysisof p53 alterations in human cancer: a review.Int J Cancer 57:1-9. 1994.

40. Barnabas N, Moraes R, Calaf G, Estrada S and Russo J: Role of p 53 in MCF10F cell immortalization and chemically induced neoplastic transformation. Int J Oncol7: 1289-1296, 1996.
41. Tait L, Soule HD and RussoJ : Ultra structural and immunocytochemical charectarization of an immortalized human breast epithelial cell line, MCF10F. Cancer Res 50:6087-6094, 1990.
42. Higgy, N.A., Salicioni, A.M., Russo, I.H., Zhang, P.L. and Russo, J. Differential expression of human ferritin H chain gene in immortal human breast epithelial MCF-10F cells. Molecular Carcinogenesis, 20:332-339,1997.
43. Miller & Champion, 1997
44. Womeodu & Bailey, 1996
45. Hughes et al. (1997).
46. Bruening, W., Giasson, B. I., Klein-Szanto, A. J., Lee, V. M., Trojanowski, J. Q., and Godwin, A. K. (2000). Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. *Cancer* **88,** 2154-63.
47. Ji, H., Liu, Y. E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B. K., Rosen, C., and Shi, Y. E. (1997). Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Res* **57,** 759-64.
48. Liu, J., Spence, M. J., Zhang, Y. L., Jiang, Y., Liu, Y. E., and Shi, Y. E. (2000). Transcriptional suppression of synuclein gamma (SNCG) expression in human breast cancer cells by the growth inhibitory cytokine oncostatin M. *Breast Cancer Res Treat* **62,** 99-107.
49. Jia, T., Liu, Y. E., Liu, J., and Shi, Y. E. (1999). Stimulation of breast cancer invasion and metastasis by synuclein gamma. *Cancer Res* **59,** 742-7.
50. Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001b). alpha-Synuclein forms a complex with transcription factor Elk-1. *J Neurochem* **77,** 239-52.
51. Iwata, A., Maruyama, M., Kanazawa, I., and Nukina, N. (2001a). alpha-Synuclein affects the MAPK pathway and accelerates cell death. *J Biol Chem* **276,** 45320-9.
52. Ridley, A. (2000). Molecular switches in metastasis. *Nature* **406,** 466-7.
53. Krueger, J. S., Keshamouni, V. G., Atanaskova, N., and Reddy, K. B. (2001). Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. *Oncogene* **20,** 4209-18.
54. Ridley, A. J. (2001). Rho GTPases and cell migration. *J Cell Sci* **114,** 2713-22.
55. Ridley, A. J., Allen, W. E., Peppelenbosch, M., and Jones, G. E. (1999). Rho family proteins and cell migration. *Biochem Soc Symp* **65,** 111-23.
56. Wicki, A., and Niggli, V. (2001). The Rho/Rho-kinase and the phosphatidylinositol 3-kinase pathways are essential for spontaneous locomotion of Walker 256 carcinosarcoma cells. *Int J Cancer* **91,** 763-71
57. Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* **103,** 239-52.
58. Rosette, C., and Karin, M. (1996). Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* **274,** 1194-7.
59. Krasilnikov, M., Adler, V., Fuchs, S. Y., Dong, Z., Haimovitz-Friedman, A., Herlyn, M., and Ronai, Z. (1999). Contribution of phosphatidylinositol 3-kinase to radiation resistance in human melanoma cells. *Mol Carcinog* **24,** 64-9.
60. Nomura, M., Kaji, A., Ma, W. Y., Zhong, S., Liu, G., Bowden, G. T., Miyamoto, K. I., and Dong, Z. (2001). Mitogen- and stress-activated protein kinase 1 mediates activation of Akt by ultraviolet B irradiation. *J Biol Chem* **276,** 25558-67.
61. Lee, L. F., Li, G., Templeton, D. J., and Ting, J. P. (1998). Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). *J Biol Chem* **273,** 28253-60.

62. Mandlekar, S., Yu, R., Tan, T. H., and Kong, A. N. (2000). Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. *Cancer Res* **60,** 5995-6000.
63. Wang, T. H., Popp, D. M., Wang, H. S., Saitoh, M., Mural, J. G., Henley, D. C., Ichijo, H., and Wimalasena, J. (1999). Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J Biol Chem* **274,** 8208-16.
64. Wang, T. H., Wang, H. S., and Soong, Y. K. (2000). Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* **88,** 2619-28.
65. Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W. A., and Chambers, T. C. (2000). Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem* **275,** 29980-5.
66. Osborn, M. T., and Chambers, T. C. (1996). Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. *J Biol Chem* **271,** 30950-5.
67. Stone, A. A., and Chambers, T. C. (2000). Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. *Exp Cell Res* **254,** 110-9.
68. Anderson, S. M., Reyland, M. E., Hunter, S., Deisher, L. M., Barzen, K. A., and Quissell, D. O. (1999). Etoposide-induced activation of c-jun N-terminal kinase (JNK) correlates with drug-induced apoptosis in salivary gland acinar cells. *Cell Death Differ* **6,** 454-62.
69. Gibson, S., Widmann, C., and Johnson, G. L. (1999). Differential involvement of MEK kinase 1 (MEKK1) in the induction of apoptosis in response to microtubule-targeted drugs versus DNA damaging agents. *J Biol Chem* **274,** 10916-22.
70. Jarvis, W. D., Johnson, C. R., Fornari, F. A., Park, J. S., Dent, P., and Grant, S. (1999). Evidence that the apoptotic actions of etoposide are independent of c-Jun/activating protein-1-mediated transregulation. *J Pharmacol Exp Ther* **290,** 1384-92.
71. Ebner, T. and B. Burchell 1993. Substrate specificities of two stably expressed human liver UDP-glucuronosyltransferases of the UGT1 gene family. Drug Metab Dispos. 21: 50-5.
72. Dodge, J.A., C.W. Lugar, S. Cho, L.L. Short, M. Sato, N.N. Yang, L.A.Spangle, M.J. Martin, D.L. Phillips, A.L. Glasebrook, J.J. Osborne, C.A.Frolik, H.U. Bryant 1997. Evaluation of the major metabolites of raloxifene as modulators of tissue selectivity. J Steroid Biochem Mol Biol. 61: 97-106.
73. Rogatko, A. and M. Slifker 1999. HWDIAG Version 1-beta.http:www.fccc.edu/users/rogatko/hwdiag_guide.html.
74. Gong, Q., J. Cho, T. Huang, C. Potter, N. Gholami, N. Basu, et al. 2001. Thirteen UDPglucuronosyltransferase genes are encoded at the human *UGT1* gene complex locus. Pharmacogenetics. 11: 357-68.
75. Albert, C., M. Vallee, G. Beaudry, A. Belanger, D. Hum 1999. The monkey and human uridine diphosphate-glucuronosyltransferase UGT1A9, expressed in steroid target tissues, are estrogen-conjugating enzymes. Endocrinology. 140: 3292-302.
76. Ciotti, M., A. Marrone, C. Potter, I.S. Owens 1997. Genetic polymorphism in the human UGT1A6 (planar phenol) UDP-glucuronosyltransferase: pharmacological implications. Pharmacogenetics. 7: 485-95.

TITLE: FOX CHASE CANCER CENTER INSTITUTIONAL BREAST CANCER TRAINING PROGRAM (FCCC-IBCTP)

PRINCIPAL INVESTIGATOR: JOSE RUSSO, M.D., F.C.A.P.

\

APPENDIX

Exhibit A: Roster of Lectures for the DOD Breast Cancer Training Program

Exhibit B: Publications enclosed:

Appendix
Exhibit A

DOD breast Cancer Training Program
Seminar Series and Dates of Activities for2001

Day	Speaker	Theme
Thursday, July 25.	Dr. Y. Matsumoto FCCC	DNA Repair and Cancer.
Thursday, August 30.	Dr. A. Godwin FCCC	Familial Breast Cancer.
Friday, September 7 Presentation by Trainees to the Faculty and Advisory Board.	Dr H. Lareef	The role of chromosome 13 in a model of breast carcinogenesis *in vitro.*
	Dr. J. Zalatoris	Functional Polymorphisms of UDP-Glucuronosyltransferase.
	Dr Z Pan.	Roles of gamma-synuclein breast and ovarian cancer.
	Dr. K. Sherman	Evaluating the efficacy of a cognitive-affective social network intervention for African.
Thursday, September 27.	Dr. R. Raftogianis FCCC	Metabolism of Estrogen and Breast Cancer.
Thursday, October 25.	Dr. C. Patriotis FCCC	From cDNA array to Proteomic.
Thursday, November 15.	Dr. E. Golemis FCCC	Addressing the Role of Complex Proteomic Networks in Cancer Related Signaling.
Thursday, December 13.	Dr. R. Strich FCCC	The Role of the Stress Response and Breast Cancer.

DOD breast Cancer Training Program
Seminar Series and Dates of Activities
For 2002

Date	Speaker	Theme
Thurs., February 14.	Dr. E. Sauter Jefferson Medical School	Approaches to the Diagnosis of Breast Cancer.
Thurs. March 14 Presentation by Trainees to the Faculty and Advisory Board.	Dr H. Lareef	Estrogen metabolites induce cell transformation in breast epithelial cells.
	Dr. J. Zalatoris	UGT pharmacogenetics and the specificity of Raloxifene.
	Dr Z Pan.	Gamma-synuclein in breast cancer.
Thursday, May 9.	Dr. G. Mor, Yale University Medical School	Life after death? Survival by apoptosis in reproductive Tissues.
Thursday, May 16.	Dr. M. Kazanietz , University of Pennsylvania	Protein Kinase C and novel receptors for the phorbol ester tumor promoters.
Friday, May 17.	D. J. Janssens	HCG treatment in a advanced breast cancer.
Thursday, June 6.	Dr. E. Henske FCCC	The tuberous sclerosis gene tumor suppressor genes activate Rho and ER regulate cell motility.
Thursday, June 20.	Dr. S. Seeholzer FCCC	How to do proteomics.
Thursday, July 25.	Dr. L. Goldstein FCCC	Clinical advances in breast cancer.
Thursday, August 22.	Dr. D. Broccoli FCCC	Telomerase and telomere in tumorigenesis.
Thursday, September 5.	Dr H. Lareef FCCC	Final report on the role of genes located in Ch 13 in the transformation of breast epithelial cells.
	Dr. J. Zalatoris FCCC	Final report on functional polymorphisms of UDP-glucuronosyltransferase.

	Dr Z. Pan FCCC	**Final report on gamma-synuclein in breast cancer.**
	Dr. K. Sherman FCCC	**Final report on Evaluating the efficacy of a cognitive-affective social network intervention for African.**
Thursday, October 24.	**Dr. A. Bellacosa FCCC**	**Defect in DNA repair and predisposition to cancer.**
Thursday, November 21.	**Dr. S. Miller FCCC**	**Psychosocial aspect in breast cancer.**
Thursday, December 12.	**Dr G. Radice, University of Pennsylvania**	**The role of cadherin in breast cancer. Its biological significance.**

TITLE: FOX CHASE CANCER CENTER INSTITUTIONAL BREAST CANCER TRAINING PROGRAM (FCCC-IBCTP)

PRINCIPAL INVESTIGATOR: JOSE RUSSO, M.D., F.C.A.P.

Appendix B.

Publications enclosed:

1-Mello, M.L.S., B.de Campos Vidal, Lareef, M.H., J.Russo. Changes in chromatin texture in transformed cells as assessed by molecular biology assays and image analysis. 14th international Congress of Cytology May 27-31 Amsterdam-The Netherland.

2-Mohamed H. Lareef, Irma H. Russo, Fathima S Sheriff, Jorge Thomas, Andrew Cuthbert, and Jose Russo. Transfer of chromosome 13 abrogates the expression of immortalization, in vitro phenotypes and tumorigenesis in human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 43:5-6, 2002.

3-Shawn Straszewski, Joon Song, Mohamed Lareef, Bozena Hanczaruk, Marijke Kamsteeg, Jose Russo and Gil Mor. The regulation of FLIP in Human Breast Epithelial Cells. Journal of the society for Gynecologic investigation supplement. 49th annual meeting Los Angeles, CA. 2002

4-Mello, M.L.S., Lareef, M.H., Vidal, B.C. and Russo, J. RNA relocation at mitosis in benz (a) pyrene transformed human breast epithelial cells after microcell mediated transfer of chromosomes 11 and 17. Analytical Cellular Pathology, 23:137-141, 2001.

5-Sherman, K., Montrone, M., Tyler, J., Driscoll, G., & Miller, S.M. (2002) The 23rd International Conference of the Stress and Anxiety Research Society. Paper on: Coping strategies, monitoring attentional style and infertility-related distress among couples undergoing in-vitro fertilization. Melbourne, Australia, July, 2002.

6-Sherman, K.A., Miller, S.M., Rodoletz, M., Driscoll, J., Daly, M., Godwin, A., & Babb, J. The 23rd Annual Meeting of the Society of Behavioral Medicine. Paper on: The role of monitoring and anticipated *BRCA1/2* carrier status on family communications intentions and plans among women with a hereditary pattern. Washington, DC, April, 2002.

7-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., & Babb, J. (2002). The 23rd Annual Meeting of the Society of Behavioral Medicine. Poster on: Enhancing Adaptation to Prostate Cancer Risk Feedback and Screening Adherence. Washington, DC, April, 2002.

8-Sherman, K., Montrone, M., Miller, S.M., Tyler, J., M.D., & Driscoll, G. (2002) The 23rd Annual Meeting of the Society of Behavioral Medicine. Poster on: Psychosocial predictors of pregnancy outcome among couples undergoing In-Vitro Fertilization. Washington, DC, April, 2002.

9-Miller, S.M., Driscoll, J., Rodoletz, M., Sherman, K., Buzaglo, J., Daly, M., Godwin, K., & Babb, J. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: Attentional style and adjustment to participation in genetic testing for inherited breast and ovarian cancer risk. Bethesda, MD, March, 2002.

10-Miller, S.M., Sherman, K., Rodoletz, M., Buzaglo, J., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: The role of monitoring and anticipated BRCA1/2 carrier status on family communication among women with a hereditary pattern. Bethesda, MD, March, 2002.

11-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., & Bruner, D. (2002) The 26th Annual Meeting of the American Society of Preventive Oncology. Poster on: Prostate Cancer Risk Assessment: Enhancing Participation and Adaptation. Bethesda, MD, March, 2002.

12-Miller, S.M., Sherman, K., Rodoletz, M., Buzaglo, J., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (2002) The 60th Annual Meeting of the American Psychosomatic Society. *Citation poster* on: The role of monitoring and anticipated BRCA1/2 carrier status on family communication intentions and plans among women with a hereditary pattern for breast/ovarian cancer. Barcelona, Spain, March, 2002.

13-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., & Babb, J. (2002). The 60th Annual Meeting of the American Psychosomatic Society. Poster on: Enhancing Adaptation to and Participation in Prostate Cancer Risk Programs. Barcelona, Spain, March.

14-Sherman, K., Montrone, M., Tyler, J., Driscoll, G., & Miller, S.M. (2002). The 60th Annual Meeting of the American Psychosomatic Society. *Press release Poster* on: Coping strategies and pregnancy outcome among couples undergoing In-Vitro Fertilization. Barcelona, Spain, March, 2002.

15-Miller, S.M., Sherman, K.A., Rodoletz, M., Brower, L., Diefenbach, M.A., Stanton, L., Bruner, D., & Knowles, J.C. (2002). The 2nd Annual Meeting of the American Academy of Health Behavior. Poster on: Enhancing Participation in and Adaptation to Prostate Cancer Risk Assessment Programs and Screening Adherence. Napa Valley, CA, March, 2002.

16-Sherman, K.A., Montrone, M., Tyler, J., Driscoll, G., Miller, S.M., & Knowles, J. (2002). The 2nd Annua Meeting of the American Academy of Health Behavior. Poster on: Psychosocial predictors of pregnancy outcome among couples undergoing In-Vitro Fertilization. Napa Valley, CA, March, 2002.

17-Miller, S.M., Sherman, K.A., Buzaglo, J.S., & Rodoletz, M. (2001). Monitoring-Blunting behavioral signatures in coping with health threats: The example of cancer. Psicologia della Salute, 3, 37-48.

18-Sherman, K.A., Miller, S.M., & Sheinfeld-Gorin, S. (Under review). Psychosocial determinants of participation in risk counselling programs and breast cancer screening regimens among African American women. Breast Cancer in African American Women Monographs, Susan G. Komen Foundation and the African American National Advisory Committee.

19-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y, and A.K. Godwin (2002). Gamma-synuclein is over-expressed in breast and ovarian cancers and promotes tumor cell survival by inhibiting stress-induced apoptosis. AACR 93rd Annual Meeting, San Francisco, April 6-10, 2002.

20-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y, and A.K. Godwin (2001). Gamma-synuclein, a candidate oncogene, activates Rac and ERK and contributes to the metastatic spread of breast and ovarian cancer. 51st Annual Meeting of the American Society of Human Genetics, San Diego, Oct. 12-16, 2001.

21-Frolov, A., Pan, Z.Z., Broccoli, D., Giaisson, B.I., Verderveer, L., Auersperg. N., Lynch, H., Daly, M., Hamilton, T. and A.K. Godwin (2001). Identification of ovarian cancer-associated genes using a HOSE cell transformation model. NCI (National Cancer Institute) 9th SPORE (Specialized Program of Research Excellence) Investigators' Workshop, Washington, DC, July 15-17, 2001.

22-Pan, Z.Z., Bruening, W., Giaisson, B.I., Lee, V.M.Y., Godwin, A.K. Gamma-synuclein promotes cancer cell survival and inhibits stress- and chemotherapeutic drug-induced apoptosis by modulating MAPK pathways. (Revised manuscript, JBC2002).

23-Frolov, A., Arnoletti, J.P., Pan, Z.Z., von Mehren, M., Eisenberg, B. and A.K. Godwin. *Sprouty* 4A, a novel genetic marker of response to imatinib mesylate in gastrointestinal stromal tumors. (Submitted, Nature Medicine 2002).

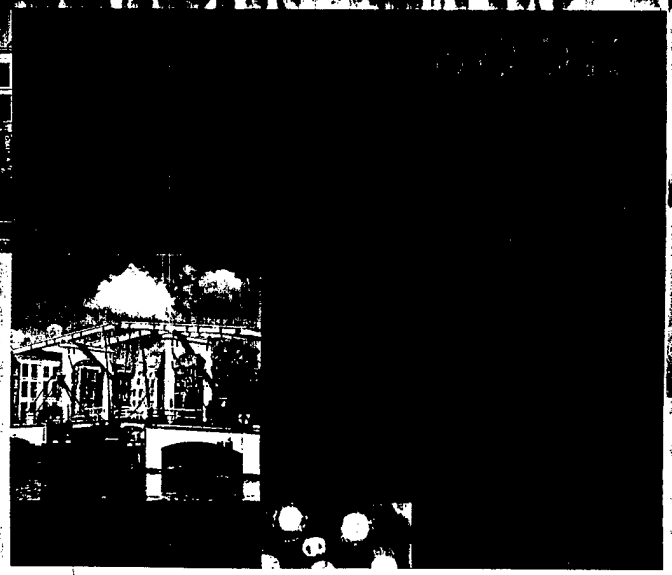
24-Prowse, A.H., Vanderveer, L., Milling, S.W.F., Pan, Z.Z., Dunbrack, R.L., Xu, X.X., and A.K Godwin (2002). OVCA2 is down-regulated and degraded during retinoid-induced apoptosis. Int. J. Cancer 99:185-192.

14th INTERNATIONAL CONGRESS OF CYTOLOGY

MAY 27 - 31 AMSTERDAM
THE NETHERLANDS

organized by
the International Academy of Cytology

ABSTRACTS



454: INTRAOPERATIVE CYTOLOGICAL DIAGNOSIS OF BREAST SPECIMENS

Mansour Mehzad; Gholamhossein Vakilzadeh. Department of Cytopathology and Surgery, Isfahan University of Medical Sciences. Ghassr Building, Gassr Square, Shamsabadi Street, Isfahan, IRAN.

The purpose of this study was evaluation of cytological method as an intraoperative procedure for rapid diagnosis of breast cancer and as an alternative for frozen sections.
Materials and Methods: 164 breast incisional biopsies were examined, the cut surface of each specimen was vigorously scraped with a scalpel blade, the material was spread on 3 to 4 glass slides, fixed in 95% alcohol for 2 minutes and stained by rapid H&E and mounted. The whole procedure lasted for 4 to 5 minutes. The surgeon was let to know whether the lesion was begin or malignant in order to decide for mastectomy.
Results: The results were compared with histological reports. 89 lesions were benign and 75 were malignant. Cytological diagnoses were correct in all cases except for one case of invasive lobular cancer with a false negative cytological report. No false positive were encountered.
Conclusion: This method has advantages of rapidity, cheapness, sparing of tissue for other studies, better correlation with FNA results and more complete sampling of large or multiple specimens. The results are as accurate as frozen sections.
Poster Session: II: May 29: 16.00 - 18.00: Poster: 120 min.

455: ENHANCED DIAGNOSIS OF CERVICAL INFECTIONS ON MONOLAYER SLIDES

Elisabeth M. Meijer-Marres1, Elisabeth Ouwerkerk-Noordam1, Mathilde E. Boon1, D. Verbeek2. (1) Leiden Cytology and Pathology Laboratory, Leiden, The Netherlands. (2) Canisius-Wilhelmina Hospital, Department of Cytology, Nijmegen, The Netherlands.

Objective: The diagnostician is not only asked to screen for (pre)malignant cells, but in addition to look for infectious agents in the cervical samples. Since in monolayer slides, the diagnostic material is deposited on the slide in a fixed state, we can expect that the morphology of the infectious agents differs from the well-known images in cervical smears. Our goal was to study how this novel preparation technology influences the morphology and staining qualities of Trichomonads, fungi, and Actinomyces.
Methods: Cases were selected in which the split-sample smear contained Trichomonads, fungi, or Actinomyces. The ThinPrep system was used for the monolayer slides.
Results: The Trichomonads were rounder, smaller, varied less in size and shape. They stained more intensely with Light Green and accordingly stood out against the clean background. Often, many Trichominads were clinging to the squamous epithelial cells. The spores of fungi stained less intensely with Eosin. The hyphl were very easy to detect because, when sticking out of the epithelial cells, they contrast with the clean background. The balls of Actinomyces were slightly loosened up but not difficult to identify. The leucocytes were mainly intraepithelial and/or attached to the epithelial cells.
Conclusion: When aware of the differences between monolayer slides and smears, the diagnosis of cervical infections is not difficult. The fixation in suspension and the filtering of the cellular sample even enhances the diagnosis.
Poster Session: I: May 28: 16.00 - 18.00: Poster: 120 min.

456: CHANGES IN CHROMATIN TEXTURE IN TRANSFORMED CELLS AS ASSESSED BY MOLECULAR BIOLOGY ASSAYS AND IMAGE ANALYSIS

M.L.S. Mello1, B. de Campos Vidal1, M.T. Lareef2, J. Russo2. (1)Institute of Biology, UNICAMP, Campinas, SP, Brazil. (2) Fox Chase Cancer Center, Philadelphia, PA, USA.

Molecular changes associated with breast cancer progression have been characterized using the MCF-10F cell series. The MCF-10F cell line was originally established from fibrous mastectomy tissue of a patient without detectable cancer. When MCF-10F cells were treated by the carcinogen benzo [a] pyrene (BP), different cell lines with gradual steps of tumoral progression (BP1, BP1-E, BP1-E1, BP1-Tras, among others) have been produced. We have been searching relationships amidst changes in DNA content, interphase cell chromatin supraorganization (defined in terms of chromatin texture), and the expression of different stages of tumorigenesis in these cells. Image analysis studies have revealed decreased nuclear areas simultaneous to an increase in chromatin higher order packing states and nucleolar areas and to DNA losses accompanying the expression of the in vitro tumorigenesis stages established in the MCF-10F cell series by J. Russo and his co-workers, with molecular biology assays. Since affected chromosomes 11 and 17 have been associated with the neoplastic progression in BP-transformed MCF-10F cells, image analysis of Feulgen-stained tumorigenic BP1-E cells subjected to microcell-mediated chromosome transfer involving unaffected chromosomes 11 and 17 was performed. The results indicated reversal of DNA amounts and nuclear sizes to a state typical of non-transformed cells, especially after the transfer of chromosome 17, whereas no reversal in chromatin texture was elicited. While chromosome 17 revealed to be primordial for normality recovery, a more complex return to genome balance is probably required for the whole chromatin to recover its normal supraorganization. (Support: FAPESP/99/2547-8, CNPq/460621/2000-9, NIH RO1CA67238)
Seminar XI: DNA, Flow and Image Cytometry: May 30: 10.00 - 12.00: Oral presentation: 15 min.

457: DNA-PLOIDY (FLOW CYTOMETRY) AND HIGH RISK HPV-TYPE INFECTION (RFLP) AS PROGNOSTIC MARKERS FOR PERSISTENCE AND PROGRESSION OF CIN I / II (PAP IIID). COMPARISON OF BOTH TESTS IN A PROSPECTIVE CLINICAL TRIAL

Peter Melsheimer1, Rudi Claes2, Magnus v.Knebel Doeberitz 2, Gunther Baster1. (1) Dept. Gyn./Obst., Universitäts-Frauenklinik, Univ. Heidelberg, Germany; (2) Div. of Molecular Diagnostics and Therapy, Univ. Heidelberg, Germany

Objective: CIN I/II (Pap IIID - Munich2 Classification) lesions may regress spontaneously without having received any form of therapy. If these lesions persist for more than one year or progresses into CIN III (Pap IVa) usually patients are being therapied. Aim of our study was to compare detection of DNA-aneuploidy and HPV high risk type infection as diagnostic tests for prediction of persistence and progression of CIN I/II lesions in a prospective clinical trial.
Method: In a prospective follow up study, 70 consecutive outdoor patients from our colposcopy unit with CIN I or CIN II lesions (Pap IIID) were followed up for at least one year. Diagnosis had to be confirmed both by cytology and by colposcopically guided punch biopsy histology. HPV-Types were analysed from the punch biopsies and the cervical swabs by restriction fragment length polymorphism (RFLP) method, a very sensitive and specific PCR based procedure. From the same punch biopsies high resolution DNA flow-cytometry was done to determine DNA ploidy status.
Results: HPV type 16 infection and the existence of aneuploid cell lines proved to be significant risk factors for CIN I/II lesions to persist or progress to CIN III in the one-year follow-up period in the same cohort of patients. The relative risks and 95% confidence intervals (CI) were 1.81 (1.44-2.76) for aneuploid cell lines and 1.74 (1.10-2.76) for HPV type 16 infection in CIN I/II lesions. As a predictive diagnostic test for CIN I/II lesions to persist or progress the specificity and positive predictive value (PPV) for aneuploid histograms were 100% (CI 73.5%-100%) and 100% (CI 86.8%-100%), respectively. The low sensitivity of 27.3% (CI 14.9%-42.8%) restricted the clinical application of the test to predict prognosis of CIN I/II, leaving 32 of 44 women with persisting or progressing CINI/II with diploid histograms. HPV type 16 positivity by FRLP had a PPV of 68.4% (CI 43.5%-87.4%) as a prognostic test. 6 of 19 HPV 16 infected women showed complete remission of the CIN lesion A combination of the two tests did not provide any additional information.
Conclusion: DNA-Aneuploidy (analysed by high resolution DNA-cytometry) was proven to be a better prognostic marker for progression or persistence of a CIN I/II (Pap IIID) lesions than HPV-High Risk Type Infection (RFLP-analysis) in patients of a university colposcopy unit.
I & P Papers II C: Gynecologic Cytology: May 29: 10.00 - 12.00: Oral presentation: 12 min.

458: HISTIOCYTES IN LYMPH NODE ASPIRATES - A DIAGNOSTIC CLUE?.

Evelina Mendonça. Laboratório de Citologia, Instituto Português de Oncologia, Lisboa, Portugal

Histiocytes in lymph node smears are commonly associated with reactive processes and are often used as the hallmark of benign disease. The type of histiocytes present in smears may be of help in differentiating the many possible entities and, in many cases, the histiocytic cell can even be the clue for a specific diagnosis. However, an excessive confidence in this feature can be misleading and be the cause of a false negative diagnosis. Histiocytes with phagocytic activity or apoptotic bodies, either isolated or forming lymphohistiocytic aggregates suggest a benign condition, such as nonspecific reactive lymph node, infectious disease, eg. leishmaniasis, or Rosai-Dorfman disease when emperipolesis is obvious. They are however also common in high grade lymphomas but, in these cases, lymphocyte morphology is quite different and makes the distinction easier. Epithelioid histiocytes are common in infectious disorders, such as toxoplasmosis or mycobacterial diseases, but they are also not rare in low grade lymphomas, enhancing the importance of careful observation and immunophenotyping of the lymphoid population. The presence of granulomas, also common in tuberculosis and other mycobacteriosis, does not rule out malignancy as well. Hodgkin's lymphoma, anaplastic T cell lymphoma and metastatic carcinoma are among the neoplasms in which epithelioid granulomas may be identified. In immunosupressed patients, tuberculosis may present a pattern of foamy macrophages in a background of inflammatory cells. This type of smear is more frequent in non specific inflammation and particularly in Langerhans cell histiocytosis, but may sometimes be seen Hodgkin's lymphoma, so histochemistry, immunophenotyping and/or electron microscopy are needed for a final diagnosis. The same is true for diagnosing the rare histiocytic malignancies, that may be recognised in smears using an adequate panel of antibodies In summary, although present in a great variety of diseases, with some cytologic pattern overlap, by a careful evaluation of both histiocyte type and morphology of the lymphoid population histiocytes may be used as a clue for differential diagnosis and appropriate selection of ancillary techniques of lymph node aspirates.
Seminar VIII: FNAC of Lymph Nodes: May 29: 10.00 - 12.00: Oral presentation: 15 min.

AACR American Association for Cancer Research

93rd Annual Meeting

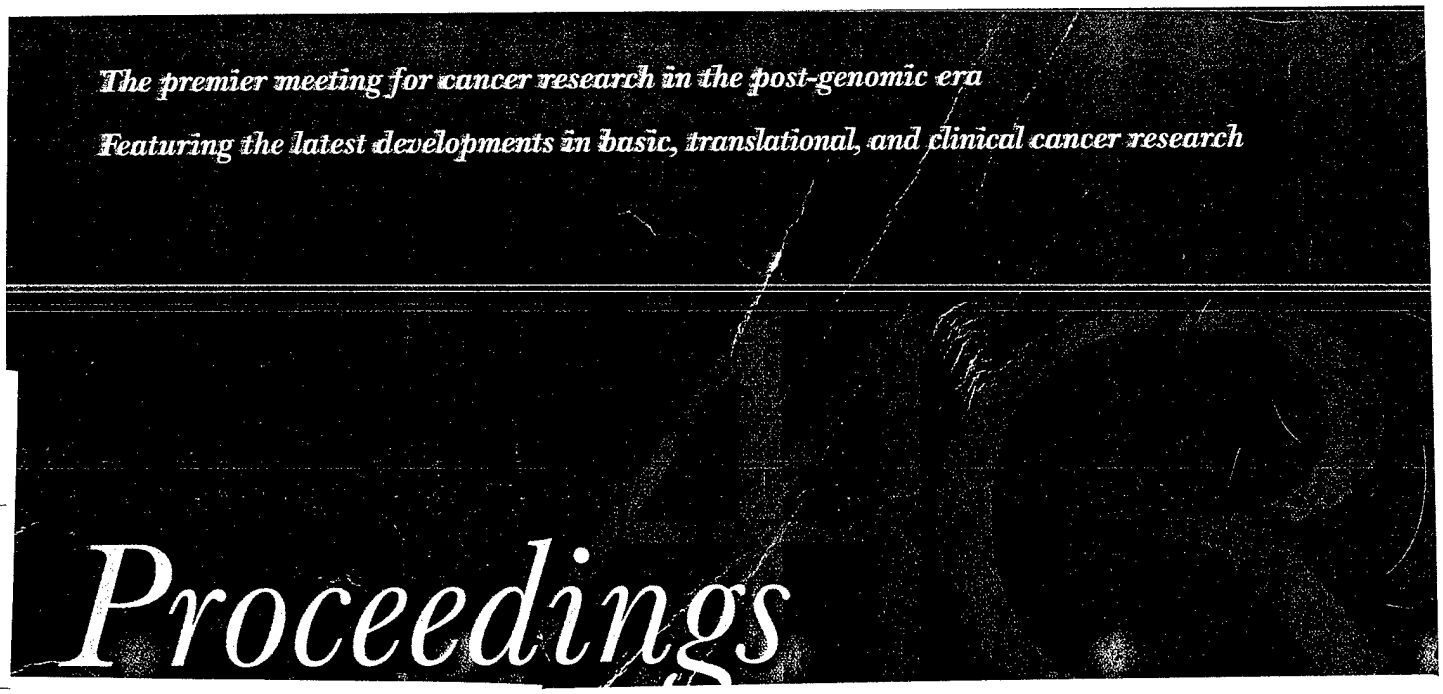
April 6-10, 2002 • San Francisco, California

Volume 43 • March 2002

In joint sponsorship with the Keck School of Medicine of the University of Southern California

The premier meeting for cancer research in the post-genomic era

Featuring the latest developments in basic, translational, and clinical cancer research



Proceedings

1577-88). We have also found that poorly differentiated and metastatic human breast tumor cells, such as MDA-MB231 and MDA-MB436, expressed high levels of Id-1, even in the absence of serum or growth factors (Cancer Res 2000 60(5):1332-40). These observations suggested that Id-1 may play a role in breast cancer progression. We also investigated the expression of another Id protein, Id-2, in mammary epithelial cells. In contrast to Id-1, we found that, *in vitro* and *in vivo*, Id-2 mRNA and protein were up-regulated as cells lost proliferative capacity and initiated differentiation (JBC 2001 276(42): 39213-9). We recently found that, upon serum withdrawal, only Id-2, and not Id-1, was expressed at a high level in the most differentiated human breast cancer cells T47D and MCF-7. In the same conditions, Id-2 was expressed at a low level in metastatic cells. Therefore, Id-2 expression seemed to follow a pattern opposite to that of Id-1 during during breast cancer cell progression. When non-aggressive T47D cells were cultured in presence of serum or estrogen alone, they expressed high levels of Id-1 and low levels of Id-2. Upon treatment with progesterone (Pg), which was shown to reduce the aggressive phenotype of progesterone receptor (PR) positive breast cancer cells, we could detect a down-regulation of Id-1 accompanied with an up-regulation of Id-2. On the other hand, Pg showed no effect on Id expressions in metastatic MDA-MB231 cells that lack PR. However, after introducing PR into MDA-MB231 cells, Pg inhibited the expression of Id-1-mRNA drastically and reduced their level of aggressiveness. These findings lead us to hypothesize that both Id-1 and Id-2 are involved in regulating breast cancer progression, but by playing opposite roles, with their expression inversely correlated. Id-2, an activator of differentiation in mammary epithelial cells may ultimately be useful as an indicator of good prognosis for breast cancer. Expression of Id-2 may be down-regulated during breast cancer progression and metastasis, and this down-regulation might be directly correlated with Id-1 up-regulation. Therefore, by targeting Id-1 in metastatic cells, we may be able to up-regulate Id-2, and more efficiently redifferentiate aggressive breast cancer cells.

#21 Alterations in cytoskeletal structure induced by the polyamine (PA) biosynthetic inhibitor α−difluoromethylornithine (DFMO) in human mammary epithelial cells. Andrea Manni, Laura Craig, Sharlene Washington, Michael Cloud, Michael F. Verderame, and David Mauger. *Pennsylvania State University, Hershey, PA.*

Activation of the PA pathway is likely to promote carcinogenesis and, therefore, inhibition of PA biosynthesis is being tested as a chemopreventive strategy. Since alterations in cytoskeletal architecture are involved in transformation, the present experiments were designed to test the effect of the PA biosynthetic inhibitor DFMO on actin polymerization and tubulin organization of MCF-10A human breast epithelial cells grown in liquid culture. F-actin staining with rhodamine phalloidin exhibited a preferential submembrane distribution in MCF-10A cells. The intensity of F-actin staining was blindly scored (1+, 2+, 3+) by five independent observers in multiple randomly selected fields per experimental conditions. We observed that administration of DFMO (in doses ranging from 0.05 to 1 mM) significantly reduced ($p=0.0012$, ANOVA) submembrane actin staining. The effect of DFMO was totally reversible with exogenous putrescine (2.5 mM), while the addition of this PA alone had no effect. Using a mouse anti−β tubulin antibody, we observed a typical perinuclear tubulin organizing center in untreated MCF-10A cells. Treatment with DFMO caused a significant ($p=0.0007$, ANOVA) dose-dependent reduction and disruption of perinuclear beta tubulin staining (quantitated similarly as described for F-actin), which were again reversible with exogenous putrescine administration. These results indicate a critical role for PA in the preservation of the cytoskeletal integrity of MCF-10A human breast epithelial cells. Alterations in cytoskeletal structure may mediate the antitumor/chemopreventive effects of DFMO in breast cancer.

#22 Laminin-5 inhibits dedifferentiation of breast epithelial cells. Reju M. Korah, Meera Hameed, Petra A. Archibald, and Robert Wieder. *UMDNJ-New Jersey Medical School, Newark, NJ.*

Using breast cancer specimens and established breast cancer cell lines we investigated the role of Laminin-5, an extracellular matrix (ECM) protein in maintaining a differentiated phenotype of breast epithelial cells. Immunohistochemical studies showed a clear association between expression pattern of Laminin-5 and the disease stage of breast cancer. In normal mammary duct, basement membrane surrounding myoepithelial and luminal epithelial cells showed high levels of Laminin-5, which declined progressively with the advancement of dedifferentiation. In specimens representing high-grade invasive breast cancer, laminin-5 expression was undetectable. To test the potential role of laminin-5 in mediating breast cancer cell differentiation, we compared malignant behavior of three well-studied breast cancer cell lines, MDA-MB-231, T-47D and MCF-7 to that of their more-differentiated FGF-2-expressing derivatives (231/F, T47D/F and MCF7/F). All three FGF-2-expressing derivatives also expressed high levels of laminin-5, which was eventually deposited in their ECM. Both short-term (less than 4 hours to avoid participation of newly deposited laminin-5) and long term (more than 8 hours that allowed the deposition of laminin-5 in cell's ECM) assays were carried out to examine a variety of malignant characteristics. In short-term assays 231/F cells showed relatively less adhesion and increased migratory potential on various ECM proteins including fibronectin, laminin-1 and collagen IV. In long-term assays on the other hand, laminin-5-expressing 231/F, T47D/F and MCF7/F cells showed a significant decrease in their potential for chemotaxis. All three laminin-5-expressing cell types (231/F, T47D/F and MCF7/F) also showed significant reduction in their capabilities to invade Matrigel and form colonies in soft agar. In addition, all three laminin-5-expressing cells formed relatively more defined branching structures in three-dimensional Matrigel cultures after six days of growth. Taken together, these associations clearly suggest a potential role for laminin-5 in blocking malignant progression of breast epithelial cells.

#23 Malignant transformation of human ovarian surface epithelial cells through genetic modifications. Jinsong Liu, Zhibo Yang, Jennifer A. Thompson, Nelly Auersperg, Gordon B. Mills, William C. Hahn, and Robert C. Bast, Jr. *The University of Texas M. D. Anderson Cancer Center, Houston, TX, University of British Columbia, Vancouver, Canada, and Dana Farber Cancer Institute, Harvard University, Boston, MA.*

To define the genetic changes involved in the development of human ovarian cancer, we serially introduced genetic elements into human ovarian surface epithelial cells (HOSE) to disrupt the pathways that are altered in human ovarian cancer. Introduction of the catalytic subunit of human telomerase (hTERT) into three HOSEs that contain SV40 large T and small t antigen immortalizes these cells *in vitro*, whereas introduction of SV40 large T and t antigen alone extends cell life but is not sufficient to immortalize. We thus introduced H-rasV12 into one of these immortalized cell lines, T-IOSE-29, to generate T-IOSE-29-H-rasV12. These cells showed a marked increase in anchorage-independent growth. No tumors were observed in 4 mice injected with parental IOSE-29 cells that contained SV40 large T and t antigens (6 subcutaneous injections and 2 intraperitoneal injection) or in 4 mice injected with T-IOSE-29 that contained both hTERT and SV40 large T and t antigen (2 peritoneal and 2 subcutaneous injections). In contrast, three of four mice that received intraperitoneal injection of T-IOSE29-H-rasV12 cells developed poorly differentiated carcinoma of the peritoneum with widespread metastasis into omentum, diaphragm, and liver similar to that seen human patients with late stage human ovarian cancer. Subcuteneous injection with T-IOSE-29-H-rasV12 cells in 100% of sites (7/7 mice) resulted in poorly differentiated carcinomas with focal papillary growth. These results demonstrated that introduction of SV40 large T and t antigen, hTERT, and H-rasV12 is sufficient to cause malignant transformation of human ovarian surface epithelial cells. Future studies of the genetic pathways disrupted by each of these components in naturally occurring human ovarian cancer may further our knowledge of its development.

#24 Transfer of chromosome 13 abrogates the expression of immortalization, *in vitro* transformation phenotypes, and tumorigenesis in human breast epithelial cells. Mohamed H. Lareef, Irma H. Russo, Fathima S. Sheriff, Jorge Thomas, Andrew Cuthbert, and Jose Russo. *Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA, Fox Chase Cancer Center, Philadelphia, PA, and Guy's, King's and St. Thomas' School of Medicine, London, UK.*

The transformation of the spontaneously immortalized human breast epithelial cell (HBEC) line MCF-10F with the chemical carcinogen benz(a)pyrene (BP) and the mutated c-Ha-ras oncogene (Int. J. Oncol. 11:41, 1997) serves as an *in vitro-in vivo* system that recapitulates the multistage process of breast cancer initiation and progression. BP-transformed cells (BP1) expressed *in vitro* the following phenotypes indicative of neoplastic transformation: colony formation in agar-methocel, expressed as colony efficiency (CE), reduced ductulogenic index (DI) and increased solid mass (SM) formation in collagen matrix, and increased invasiveness in Matrigel chambers, or invasive index (INVI). Ras transfected BP1 cells (BP1Tras) expressed, in addition, tumorigenesis in severe immunodeficient (SCID) mice. From BP1Tras-induced tumors three cell lines, BP1Tras-T1, BP1Tras-T2, and BP1Tras-T3 were originated. The three cell lines expressed microsatellite instability (MSI) and loss of heterozygosity (LOH) in several loci of chromosome 13 (Mol. Carcinog., 24:118, 1999). In order to test the functionality of these genomic alterations, the chromosome 13 was transferred to the three cell lines using microcell mediated chromosome transfer technique and cell selection by culture in hygromycin. Hybrids sub-cloned from BP1Tras-T1 and T-2 cells became senescent after the 5th and 6th passages, respectively, an indication that both transformation and immortalization phenotypes were abrogated in these cell lines. BP1Tras-T3 derived clones, instead, were continuously passaged after chromosome transfer. Eight of these clones expressed the immortalization phenotype, increased CE, and altered ductulogenic pattern with decreased DI at the expense of SM formation, resulting in decreased DI:SM ratio, like the parental BP1Tras-T3 cells. Two of these clones, however, did not induce tumors in SCID mice, and six of them formed very small (6.4mm3) tumors that were slow growing and less aggressive than those produced by the parental BP1Tras-T3 cells, which measured 140.5 mm3. These data indicate that the transfer of chromosome 13 reestablished those loci that were altered in the parental cells, successfully abolishing the expression of the *in vitro* and *in vivo* transformation phenotypes in clones BP1Tras-T1 and BP1Tras-T2, and inhibiting the expression of the tumorigenic phenotype in BP1Tras-T3 cells in which the immortalization and transformation phenotypes were retained. (Supported by grants NIH CA-RO1 67238, DAMD 17-00-1-0247 and DAMD 17-00-1-0249).

The Regulation of FLIP in Human Breast Epithelial Cells

Shawn Straszewski[1], Joon Sung[2], Mohamed Lareef[3], Bozena Hanczaruk[2], Marijke Kamsteeg[2], Jose Russo[3] and Gil Mor[2]

[1]Department of Molecular, Cellular and Developmental Biology, [2]Department of Obstetrics and Gynecology; Yale University School of Medicine; New Haven, CT
[3]Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philidelphia, PA

Objective: In a previous study, we determined that chemically transformed human breast epithelial cells transfected with chromosome 17p13.2 undergo phenotypic reversion and become sensitive to Fas-mediated apoptosis. Both normal and cancerous cells expressed similar levels of Fas and FasL, suggesting that the change in sensitivity was due to the alteration of one or more intracellular components of the Fas pathway. In this study, we demonstrate that sensitivity to Fas-mediated apoptosis is associated with the modulation of FLIP activation.

Methods: Apoptosis was induced in the cell lines by either serum deprivation or with an anti-Fas monoclonal antibody for various time periods and evaluated with CellTiter 96 assay. The expression levels of Fas, FasL, DAP Kinase, Caspase-3, Caspase-8 and FLIP were determined using RT-PCR and Western Blot analysis following Fas stimulation. In addition, Caspase-3 activity was measured with the CaspACE assay to confirm whether the cells were undergoing apoptosis.

Results: Normal breast epithelial cells and the cell lines that regained the lost allele by chromosome 17 transfection were sensitive to Fas-mediated apoptosis and did not express $FLIP_C$, the active form of FLIP. The cancerous cells and the cell lines that displayed loss of heterozygosity, on the other hand, expressed high levels of $FLIP_C$ and were resistant to Fas-mediated apoptosis. Moreover, Caspase-3 activity was elevated only in the cells that were sensitive to Fas-mediated apoptosis. Similar expression of Fas, FasL, DAP Kinase, $FLIP_L$ and the pro-active forms of Caspase-3 and Caspase-8 was observed in all cell lines.

Conclusion: As an antagonist of Caspase-8, FLIP blocks the activation of the Fas pathway and confers resistance to apoptosis. Previous studies have demonstrated a correlation between FLIP activation and neoplastic transformation. The present study reveals the existence of a FLIP regulatory factor, which is absent in cancerous cells and determines sensitivity to Fas-mediated apoptosis. Since transfection with chromosome 17p13.2 reverses the resistance of chemically transformed cells to Fas-mediated apoptosis, this region of the chromosome may contain a genetic factor that controls the activation of FLIP.

RNA relocation and persistence of nucleolus-like bodies at mitosis in benzo[a]pyrene-transformed human breast epithelial cells after microcell-mediated transfer of chromosomes 11 and 17

Maria Luiza S. Mello [a,*], Mohamed H. Lareef [b], Benedicto C. Vidal [a] and Jose Russo [b]

[a] *Department of Cell Biology, Institute of Biology, UNICAMP, 13084-971 Campinas, SP, Brazil*
[b] *Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA 19111, USA*

Received April 2001
Accepted 8 January 2002

RNA relocation and the incidence of nucleolus-like bodies accumulated during mitosis were studied cytochemically in benzo[a]pyrene (BP)-transformed human breast epithelial MCF-10F cells after microcell-mediated transfer of normal chromosomes 11 and 17. The changes resulting from the transfer of these two chromosomes in tumorigenic MCF-10F cells (BP1-E cell line) were examined, since alterations in these chromosomes are involved in the expression of the transformed and tumorigenic phenotypes in the MCF-10F cell series. In addition, the frequency of nucleolus-like bodies decreases drastically with transformation and tumorigenicity in MCF-10F cells, thus being conceivable that it would be affected in presence of normal chromosomes 11 or 17. The pattern of RNA relocation associated with the mitotic spindle did not vary in the cell lines analyzed. The introduction of chromosome 17 in BP1-E cells either decreased or did not affect the frequency of persistent nucleolus-like bodies. In contrast, in cells which received a normal chromosome 11, the frequency of nucleolus-like bodies was closer to that of non-transformed MCF-10F cells. These results suggest that a normal chromosome 11 but not chromosome 17 contributes to the maintenance of an RNA surplus which accumulates in nucleolus-like bodies during cell division of the human breast epithelial cells, at least *in vitro*. Some loci which were retained in the BP1-E cells which received a normal chromosome 11 are probably involved with the control of RNA transcript production.

Figure 1 on http://www.esacp.org/acp/2001/23-3_4/mello.htm.

Keywords: RNA, nucleolus-like bodies, human breast epithelial cells, microcell-mediated chromosome transfer, chromosome 11, chromosome 17

*Corresponding author. Fax: +55 19 37887821; E-mail: mlsmello@unicamp.br.

1. Introduction

When transformed with benzo[a]pyrene (BP), human breast epithelial MCF-10F cells cultured *in vitro* produce cell lines which show gradual stages of tumoral progression (BP1, BP1-E, BP1-E1 among others) [3]. RNA relocation associated with the mitotic spindle fibers does not vary during cell division in transformed MCF-10F cell series [10], as shown cytochemically using a variant of the critical electrolyte concentration assay [8]. However, the frequency of RNA-containing nucleolus-like bodies which persist during mitosis, and which were primarily described in non-transformed MCF-10 cells and assumed to be due to a non-profitable surplus of RNA [8], decreases drastically in transformed and tumorigenic MCF-10F cells [10]. In contrast, a significant increase in nucleolar size and rRNA production in interphase nuclei occurs in the BP1-E and BP1-E1 tumorigenic cell lines relative to non-tumorigenic, transformed cell line BP1 and non-transformed MCF-10F cells [1]. These findings have been explained in terms of an improved use of RNA transcripts during cell transformation and tumorigenesis, at least under *in vitro* conditions [10].

Analytical Cellular Pathology 23 (2001) 137–141
ISSN 0921-8912 / $8.00

Microsatellite instability markers in chromosomes 11 and 17 have been associated with neoplastic progression in BP-transformed MCF-10F cells [6]. Microsatellite instability may provoke defects in DNA replication or mismatch repair mechanisms [2,12,14]. Although microsatellite instability is not so commonly found in breast tumors as in colorectal cancer [7], cases have been reported in which microsatellite instability was considered an early event during human breast carcinogenesis [15,17]. Recently, microsatellite instability has been correlated with hormonal deregulation in the progression of breast cancer [4] and the cause of loss of an apoptotic pathway in ductal breast carcinomas [13]. Nearly 20% of microsatellite markers have been revealed in 33% of patients with primary breast tumors studied by Wild and co-workers [18], provided small tumor areas were removed by precise laser-microdissection. There are also reports indicating that in 11.9% of sporadic breast cancers, microsatellite instability was correlated with more advanced disease and relatively poorer prognosis [16].

When normal chromosomes 11 and 17 were transferred to tumorigenic BP1-E cells by microcell-mediated chromosome transfer (MMCT) assays, a functional role of these chromosomes in the expression of the BP1-E transformed phenotypes was demonstrated [19]. Some of the tumorigenic BP1-E cell characteristics which reverted to those of non-transformed MCF-10F cells by transfer of normal chromosomes 11 and 17 were: decrease in cell growth, reduced colony efficiency and colony size [19], and a tendency to restoration of the DNA amount and nuclear sizes to the distribution patterns typical of the non-transformed cells [11]. Telomerase activity was significantly reduced by chromosome 17 insertion [19]. It is thus conceivable that the pattern of RNA distribution and of the incidence of nucleolus-like bodies during mitosis under the same above-cited experimental conditions may also be affected. In the present study, RNA relocation and the incidence of nucleolus-like bodies during cell division were studied cytochemically in tumorigenic, BP-transformed MCF-10F cells receiving normal chromosomes 11 and 17 through MMCT assays.

2. Materials and methods

2.1. Cells

BP1-E, a BP-transformed cell line derived from MCF-10F cells currently maintained in the Breast Cancer Research Laboratory of the Fox Chase Cancer Center in Philadelphia as reported previously [3], was used in the transfer of normal chromosomes 11 and 17 by microcell-mediated chromosome transfer (MMCT). BP1-E cells were transfected with the plasmid pSV2neo using the Calphos maximizer transfection protocol (Clontech, Palo Alto, CA) and then fused with microcells generated from human chromosome donor cells (A9-11neo or A9-17neo) to produce the microcell hybrids BP1E-11neo and BP1E-17neo, respectively. Colonies surviving in DMEM medium containing G-418 (400 μg/ml) were subcloned. Four expandable clones (subclones) containing normal chromosomes 11 and 17 were used. BP1-E cells at passage 45, and BP1E-11neo and BP1E-17neo cells at passage 7 were used. The cells were grown for 48 h (BP1-E, BP1E-11neo and BP1E-17neo) or 96 h (BP1E-17neo) on well slides and fixed. Since BP1-E cells and the clone with transferred chromosome 11 grew faster than the clone transferred with chromosome 17, a confluence value of 80% was attained by these cells at different growth times (BP1-E and BP1E-11neo, 48 h; BP1E-17neo, 96 h).

2.2. Cell preparations and staining procedure

The cells were fixed in an ethanol-acetic acid mixture (3 : 1, v/v) for 1 min, rinsed in 70% ethanol for 3–5 min, and air dried before the identification of RNA by a variant of the critical electrolyte concentration (CEC) assay, which used toluidine blue (TB) and Mg^{2+} ions as competitors for the substrate binding sites. At the DNA CEC point, DNA metachromasy (violet colour) is abolished, whereas RNA metachromasy remains unchanged ($CEC_{RNA} > CEC_{DNA}$) [9]. Briefly, the cells were stained with 0.025% TB (Merck) solution in McIlvaine buffer at pH 4.1 for 15 min and then treated with a 0.05 M aqueous solution of $MgCl_2$ for 15 min. The slides were then rinsed in distilled water, air-dried, cleared in xylene and mounted in Canada balsam [9]. Preparations treated with a 0.01% RNase III (Sigma) aqueous solution for 1 h at 37°C prior to the CEC assay were used as controls. The slides were examined and photomicrographed with a Zeiss Axiophot II microscope.

2.3. Mitosis counting

Three slides corresponding to six wells of each cell line (or clone) were examined for mitotic cells. Nearly 70 dividing cells were chosen randomly from each

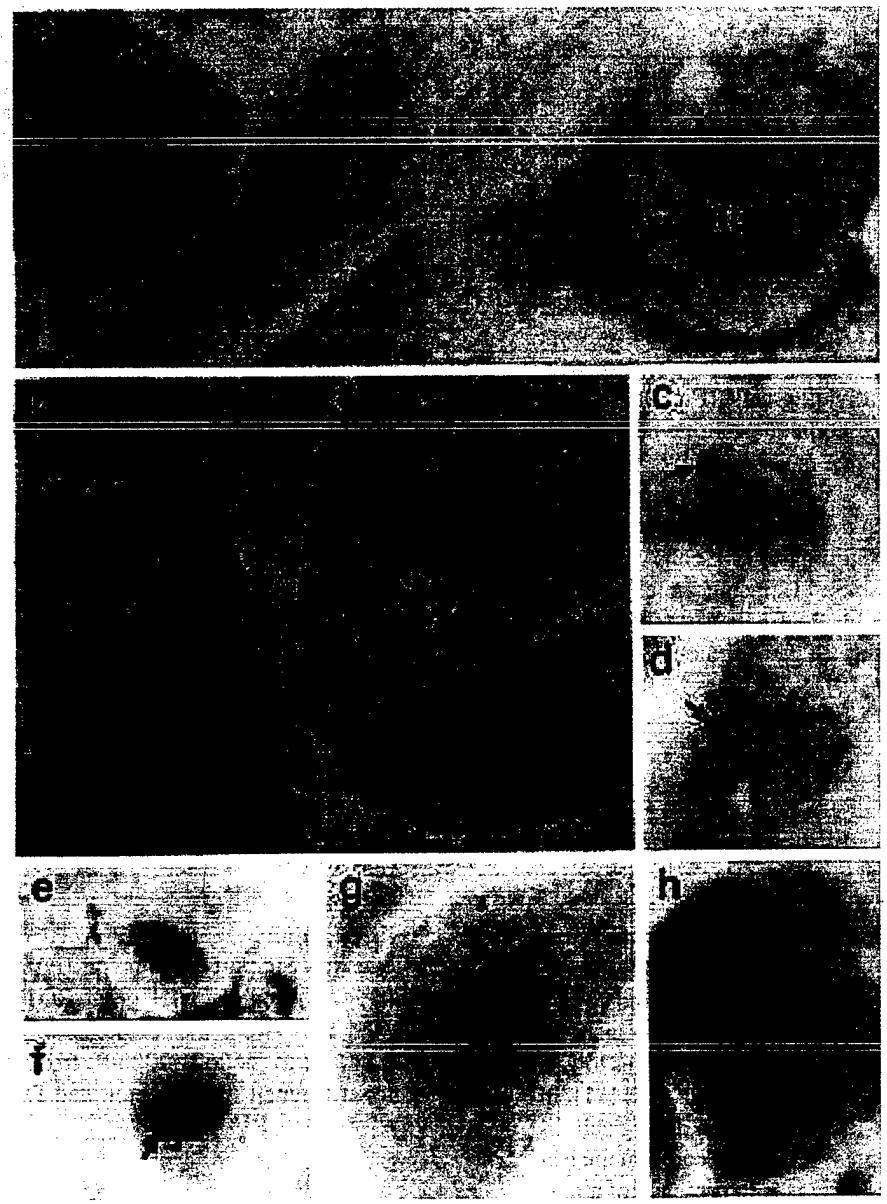


Fig. 1. Metachromatic staining of interphase cell nucleoli (nu) (a, b), nucleolus-like bodies (arrows) (a–d, f, g), and RNA along the mitotic spindle (a, g, h) in MCF-10F cell lines. e, h: cells devoid of conspicuous nucleolus-like bodies; ch: chromosomes. a–d, g, h, ×1800; e, f, ×350. This figure can be viewed on http://www.esacp.org/acp/2001/23-3_4/mello.htm.

slide and classified based on absence or presence of nucleolus-like bodies. In the case of BP1E-17neo cells grown for 48 h, the number of dividing cells analyzed per well was 25.

3. Results

RNA was identified by its metachromatic staining which could be prevented by pretreatment with RNase [9]. This staining was concentrated in the nucleoli of interphase cell nuclei (Fig. 1a, b) or lined the chromosomal mass which migrated to the equatorial plate of mitotic cells (Fig. 1a, b, e, f) and then interspaced the sets of chromosomes moving to the cell poles during anaphase/telophase (Fig. 1g, h). The pattern of RNA relocation associated with the mitotic spindle was the same in all cells examined and agreed with previous data [10].

Table 1
Incidence of nucleolus-like bodies

Cells	Cell culture		Metaphases	
	Growth (h)	Confluence (%)	No. of cells counted	Nucleolus-like bodies frequency (%)
BP1-E	48	80	218	16
BP1E-11neo	48	80	221	74
BP1E-17neo	48	50	150	6.7
	96	80	246	19.5

When nucleolus-like metachromatic bodies were present, they appeared close to the chromosomes or to the mitotic spindle in dividing cells (Fig. 1a–d, f, g). The frequency of these bodies in mitotic cells of the various cell lines is shown in Table 1. This frequency was low in BP1-E cells which received a normal chromosome 17, even when the level of culture confluence was the same as that of cultured BP1-E cells with transferred normal chromosome 11. In cells which received a normal chromosome 11, the frequency of nucleolus-like bodies was closer to that of non-transformed MCF-10F cells (86%) [10].

4. Discussion

Figure 1 can be viewed on http://www.esacp.org/acp/2001/23-3_4/mello.htm.

The transfer of normal chromosomes 11 and 17 into tumorigenic BP1-E cells has been reported to result in a 50% and 90% inhibition of cell growth, respectively, and to reduce both colony efficiency and colony size [19]. Additionally, it has been found to increase cell population DNA amounts and nuclear sizes to values typical of non-transformed MCF-10F cells [11]. In terms of the nucleolus-like bodies seen during cell division in BP1-E cells, present data indicate that the frequency of these bodies increases in presence of a normal chromosome 11, becoming closer to that of non-transformed MCF-10F cells [10]. It is thus assumed that specific regions of chromosome 11 play a functional role in the expression of phenotypes characteristic of tumoral progression in human breast epithelial cells *in vitro*, including those related to the control of RNA transcript production which is probably concerned with the accumulation of an RNA surplus during cell division.

Microsatellite polymorphism analysis has revealed that the loci 11q13–23, 11q23.1, 11q23.3, and 11q25 are retained in the BP1-E cells which receive a normal chromosome 11 through MMCT techniques [19]. Data from human genome maps in the web [5] reveal that the splicing factor 3b, subunit 2, 145 kD (SF3B2), a spliceosome-associated protein which participates in anchoring of U2 snRNP to pre-mRNA, the RNA binding motif protein 14, the breast cancer metastasis-suppressor 1 (BRMS1), and the tumor suppressor 14 (ST14) are encoded in these regions and possibly related to the above-mentioned characteristics of the BP1-E cells with transferred normal chromosome 11.

The higher frequency of nucleolus-like bodies in BP1E cells which received a normal chromosome 11 may reflect a certain poorly controlled RNA transcript production *in vitro*. This would lead to an RNA surplus accumulation during cell division in a response similar to that in non-transformed MCF-10F cells [10]. With the gradual advancing of tumorigenesis in transformed MCF-10F cells, alterations in chromosome 11 may lead cells to improve their use of RNA transcripts.

The unchanged pattern of the RNA relocation in association with the mitotic spindle architecture during cell division under present experimental conditions indicates that this process does not require normal chromosomes 11 or 17 to be effective.

Acknowledgements

This investigation was supported by the State of São Paulo Research Support Foundation (FAPESP, grant no. 99/02547-8) and by NIH grant RO1 CA67238. MLSM and BCV were the recipients of fellowships and of a grant (no. 460621/2000-9) from the Brazilian National Council for Research and Development (CNPq).

References

[1] L.F. Barbisan, J. Russo and M.L.S. Mello, Nuclear and nucleolar image analysis of human breast epithelial cells transformed by benzo[a]pyrene and transfected with the c-Ha-*ras* oncogene, *Analyt. Cell. Pathol.* 16 (1998), 193–199.

[2] J.C. Boyer, A. Umar, J.I. Risinger, J.R. Lipford, M. Kane, S. Yin, J.C. Barrett, R.D. Kolodner and T.A. Kunkel, Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines, *Cancer Res.* 55 (1995), 6063–6070.

[3] G. Calaf and J. Russo, Transformation of human breast epithelial cells by chemical carcinogens, *Carcinogenesis* 14 (1993), 483–492.

[4] T. Caldes, P. Perez-Segura, A. Tosar, M. de la Hoya and E. Díaz-Rubio, Microsatellite instability correlates with negative expression of estrogen and progesterone receptors in sporadic breast cancer, *Teratog., Carcinog. Mutagen.* **20** (2000), 283–291.

[5] http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=11 (December, 2001).

[6] Y. Huang, B. Bove, Y. Wu, I.H. Russo, Q. Tahin, X. Yang, A. Zekri and J. Russo, Microsatellite instability during the immortalization and transformation of human breast epithelial cells *in vitro*, *Mol. Carcinog.* **24** (1999), 118–127.

[7] S.C. Lee, K.D. Berg, M.E. Sherman, C.A. Griffin and J.R. Eshleman, Microsatellite instability is infrequent in medullary breast cancer, *Am. J. Clin. Pathol.* **115** (2001), 823–827.

[8] M.L.S. Mello, Relocation of RNA metachromasy at mitosis, *Acta Histochem. Cytochem.* **28** (1995), 149–154.

[9] M.L.S. Mello, B.C. Vidal, M.M. Dantas and A.L.P. Monteiro, Discrimination of the nucleolus by a critical electrolyte concentration method, *Acta Histochem. Cytochem.* **26** (1993), 1–3.

[10] M.L.S. Mello, L.F. Barbisan, J. Russo and B.C. Vidal, RNA relocation at mitosis in transformed and tumorigenic human breast epithelial cells, *Cell Biol. Int.* **23** (1999), 125–128.

[11] M.L.S. Mello, B.C. Vidal, M.T. Lareef and J. Russo, Changes in chromatin texture in transformed cells as assessed by molecular biology assays and image analysis. 14th Int. Congr. Cytology, Amsterdam, 2001, *Abstract Book*, 2001, p. 198.

[12] I. Mellon, D.K. Rajpal, M. Koi, C.R. Boland and G.N. Champe, Transcription-coupled repair deficiency and mutations in human mismatch repair genes, *Science* **272** (1996), 557–560.

[13] O. Mendez, S. Manas, M.A. Peinado, A. Fabra, A. Escobedo, A. Moreno and A. Sierra, Microsatellite instability is associated with the loss of apoptosis in ductal breast carcinomas, *Breast Cancer Res. Treatm.* **65** (2001), 171–177.

[14] E.A. Sia, S. Jinks-Robertson and T.D. Petes, Genetic control of microsatellite stability, *Mut. Res.* **383** (1997), 61–70.

[15] S.P. Siah, D.M. Quinn, G.D. Bennett, G. Casey, R.L.P. Flower, G. Suthers, Z. Rudzki and Z. Rudski, Microsatellite instability markers in breast cancer: a review and study showing MSI was not detected at 'BAT25' and 'BAT26' microsatellite markers in early-onset breast cancer, *Breast Cancer Res. Treatm.* **60** (2000), 135–142.

[16] S. Tomita, S. Deguchi, T. Miyaguni, Y. Muto, T. Tamamoto and T. Toda, Analyses of microsatellite instability and the transforming growth factor-beta receptor type II gene mutation in sporadic breast cancer and their correlation with clinicopathological features, *Breast Cancer Res. Treatm.* **53** (1999), 33–39.

[17] T. Toyama, H. Iwase, H. Iwata, Y. Hara, Y. Omoto, M. Suchi, T. Kato, T. Nakamura and S. Kobayashi, Microsatellite instability in *in situ* and invasive sporadic breast cancers of Japanese women, *Cancer Lett.* **108** (1996), 205–209.

[18] P. Wild, R. Knuechel, W. Dietmaier, F. Hofstaedter and A. Hartmann, Laser microdissection and microsatellite analyses of breast cancer reveal a high degree of tumor heterogeneity, *Pathobiology* **68** (2000), 180–190.

[19] X. Yang, Q. Tahin, Y.F. Hu, I.H. Russo, B.R. Balsara, D. Mihaila, C. Slater, J.C. Barrett and J. Russo, Functional roles of chromosomes 11 and 17 in the transformation of human breast epithelial cells *in vitro*, *Int. J. Oncol.* **15** (1999), 629–638.

COPING STRATEGIES, MONITORING ATTENTIONAL STYLE AND INFERTILITY-RELATED DISTRESS AMONG COUPLES UNDERGOING IN-VITRO FERTILIZATION

Kerry A. Sherman[1], Ph.D., Miranda Montrone[2], M.A., John Tyler[2], M.D., Geoff Driscoll[2], M.D., & Suzanne M. Miller[1], Ph.D.
[1]Fox Chase Cancer Center
Department of Behavioral Medicine, P1103
7711 Burholme Avenue,
Philadelphia, PA 19111, USA.
Email: ka_Sherman@fccc.edu
[2]City West I.V.F., Sydney, AUSTRALIA.

This study explored the relationship between patient coping styles and infertility-related distress among Australian couples entering an In-Vitro-Fertilization treatment program. Both members of medically-diagnosed infertile couples (N = 81; female: mean age 33; male: mean age 34) provided demographic information and completed the Brief COPE and Revised Impact of Events Scales. Participants were further categorized into *high monitors* (who attend to health threats) and *low monitors* (who distract from health threats). Analysis of variance indicated that women reported greater infertility-related intrusion ($p<.0001$) and avoidance ($p<.05$) than men. Distress was correlated with self-destructive behavior, substance abuse, venting, self-blame, denial and behavioral disengagement for women, and denial, self-destructive behavior, behavioral disengagement, active coping, and emotional support for men. Additionally, high monitoring was associated with intrusion for both men and women. For intrusion, self-blame ($\beta = .80$, $p<.05$) and substance abuse ($\beta = 1.06$, $p<.06$) accounted for 31.6% of the variance in women's scores, and self-destructive behaviors ($\beta = .39$, $p<.01$) and high monitoring ($\beta = .23$, $p<.05$) accounted for 23.1% of the variance in men's scores. Findings indicate the importance of tailoring communications to individual coping responses and attentional style of both partners when designing infertility treatment counseling programs.

The 23rd International Conference of the Stress and Anxiety Research Society

Paper Session #31 10:45 a.m.–11:00 a.m.

SHORTER MOMENTS OF PRIMARY NEGATIVE AFFECT ASSOCIATED WITH FLATTENED DIURNAL SLOPE OF CORTISOL IN METASTATIC BREAST CANCER PATIENTS

Janine Giese-Davis, Ph.D., Sandra Sephton, Ph.D., and David Spiegel, M.D.

Past research has found associations between shorter cancer survival and suppressed or repressed negative affect. In a prior study we found that a flattened diurnal slope of cortisol assessed prior to randomization in a clinical trial of supportive-expressive group therapy was strongly predictive of early death and was associated with repression (scored using the Weinberger Adjustment Inventory). In the current study, we investigated whether women who were less behaviorally expressive of primary negative affect during their initial group therapy session had flattened cortisol slopes. We emotion-coded the first group session of 28 women utilizing the Specific Affect Coding System. Each tape was double coded and passed a kappa above .60. Women taking steroids and megasterone were selected out due to confounds. We conducted a regression controlling for speaking time in the first step, and using simultaneous entry of Primary Negative Affect (fear, sadness, and direct anger) and Positive Affect (genuine humor, excitement, affection, validation, and interest) as independent variables. Those women who expressed less Primary Negative Affect in their first session of therapy had significantly flatter diurnal cortisol slopes ($F(3,17) = 3.22$, $p < .05$. Adjusted $R2 = .25$). Primary Negative Affect ($= -.69$, $t = -3.11$, $p = .006$), Positive Affect ($= -.20$, $t = -.96$, $p = .35$), Speaking time ($= .25$, $t = 1.21$, $p = .24$). In the first session, women often tell their cancer stories with a great deal of emotional expression. Women who do not express negative affect when it might be expected, may be at greater physiological risk.

CORRESPONDING AUTHOR: Janine Giese-Davis, Ph.D., 401 Quarry Rd., Rm. 2318, Stanford, CA 94305-5718

Paper Session #31 11:00 a.m.–11:15 a.m.

IMPACT OF CATASTROPHIZING ON FATIGUE SEVERITY AND DISRUPTIVENESS DURING BREAST CANCER TREATMENT

Christina L. Thors, Ph.D., Krista Varkey, B.A., Michael A. Andrykowki, Ph.D., and Paul B. Jacobsen, Ph.D., H. Lee Moffitt Cancer Center, University of South Florida and University of Kentucky College of Medicine

Fatigue is one of the most frequent and distressing symptoms experienced by cancer patients. Although their disease and treatment are major factors contributing to patients' experience of fatigue, psychological factors may also play a role. To evaluate this possibility, we tested the hypothesis that greater reliance on the cognitive coping strategy of catastrophizing would be associated with greater severity and disruptiveness of fatigue over the course of breast cancer treatment. Eighty-one women (mean age = 55 years) with Stage I or II breast cancer being treated with chemotherapy ($n = 43$) or radiotherapy ($n = 38$) were assessed before the start of treatment and at their final treatment. As expected, greater use of catastrophizing before treatment (measured using the Fatigue Catastrophizing Scale) was significantly ($p < .01$) associated with greater severity and disruptiveness of fatigue at the end of treatment (measured using the Fatigue Symptom Inventory). Moreover, catastrophizing remained a significant ($p = .013$) predictor even after controlling statistically for severity and disruptiveness of fatigue measured before the start of treatment. Additional analyses indicated significant ($p<.05$) interactions between catastrophizing and type of treatment; catastrophizing appeared to have a greater impact on fatigue severity and disruptiveness for radiotherapy patients. These results demonstrate the negative impact of catastrophizing on the experience of fatigue, and suggest the need for development and evaluation of interventions designed to modify this form of coping in patients about to undergo cancer treatment.

CORRESPONDING AUTHOR: Paul B. Jacobsen, Ph.D., MOD3-PSY, University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612

Paper Session #31 11:15 a.m.–11:30 a.m.

ADHERENCE TO A WRITTEN EMOTIONAL EXPRESSION INTERVENTION AMONG BREAST CANCER PATIENTS

Lee Ellington, Ph.D.*, University of Utah,1 Lillian Nail, Ph.D., Oregon Health Sciences University,2 Lee Walker, PhD,1 Motomi Mori, PhD,2 and Yi-Ching Hsieh, MS2

There has been wide interest in the use of emotional expression interventions (EEI), particularly the use of expressive writing (Pennebaker), to promote adjustment to stressors. Few EEI studies have examined adherence to writing in relation to patient characteristics, mode of intervention delivery (take-home), or outcomes. Our randomized trial comparing EEI, Concrete Objective Information (COI), and Controls on adjustment following radiation treatment (RT) for breast cancer (BC), provides an opportunity to examine longitudinal differences among the groups including adherence/nonadherence to EEI.

259 BC patients participated . They were predominantly middle-aged (M=55), Caucasian (82%), and married (66%). Subjects were assigned to 3 conditions. The attrition rates were as follows: COI and Controls (2%); EEI (31% were nonadherent). For 6 months following RT, subjects rated disclosure (DIS) about BC, positive affect (PANAS State), and confidence in dealing with post RT experiences. Using generalized linear mixed models, the EEI-writers were found to be more confident than Controls and EEI-nonwriters ($F(3, 253) = 4.74$, $p < .01$). For DIS, an interaction effect indicated that EEI-nonwriters disclosed less at baseline but at 6 months, the EEI-nonwriters reported increased disclosure ($F(9, 253)$ 2.12, $p = .03$). Additionally, the COI, Controls, and EEI-writers reported consistent positive mood; whereas, the EEI-nonwriters reported a decrease at 4 weeks which did not return to baseline levels ($F(9, 253) = 2.12$, $p = .03$).

For BC survivors, take-home EEI shows adherence rates similar to lab studies and led to increased confidence for writers. Our findings suggest that EEI is cost-effective and beneficial. Characteristics of EEI-nonwriters will be discussed.

CORRESPONDING AUTHOR: Lee Ellington, Ph.D., College of Nursing, University of Utah, 10S 2000E Front, Salt Lake City, UT 84112-5880

Paper Session #31 11:30 a.m.–11:45 a.m.

THE ROLE OF MONITORING AND ANTICIPATED BRCA1/2 CARRIER STATUS ON FAMILY COMMUNICATION INTENTIONS AND PLANS AMONG WOMEN WITH A HEREDITARY PATTERN

K. Sherman, S.M. Miller, M. Rodoletz, J. Driscoll, J. Buzaglo and M. Daly, Fox Chase Cancer Center and City West I.V.F.

This study explored the role of anticipated carrier status and attentional style on intentions and plans for communicating BRCA1/2 test results among women with a putative hereditary pattern of breast/ovarian cancer. Women (N=196; 95% Caucasian; mean age 49; 59% unaffected with breast/ovarian cancer) provided demographic information and completed the Monitor-Blunter Style Scale prior to receiving cancer risk counseling (baseline). Information was also obtained on intentions to communicate risk feedback to family members at baseline and one-week following the donation of blood for genetic testing. At baseline, most participants were certain they would communicate results to their family (71%) and children (66%), independent of attentional style and potential outcome. Participants were more inclined to seek advice about ($p<.01$), and carefully plan for ($p<.01$), communication of positive (versus negative) test results. However, even when anticipating positive results, only 41% reported serious intentions to seek advice about or carefully plan their communication. Analysis of variance indicated that low monitors (who distract from threat-related cues) reported significantly less intentions to communicate positive test results to family members ($p<.01$), and were less likely to engage in strategic planning ($p<.05$) one-week post-blood draw than high monitors (who scan for disease-related threats). Results suggest that women are motivated to share their carrier status with their family, particularly in the case of a positive result, but do not seek advice or strategically prepare for disclosure of test feedback. Attentional style should be considered when designing genetic testing counseling programs.

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111

E-86

HOW IS AGE RELATED TO OUTCOMES FOLLOWING BREAST CANCER DIAGNOSIS?

Ann K. Sandgren*, Ph.D., Roger Maris Cancer Center; Kevin D. McCaul, Ph.D., Emily O. Sandgren, Kathleen M. Romanek, B.A., and Shannon C. Erickson, B.A., North Dakota State University

We examined relationships between age and coping with breast cancer. Previous research has produced conflicting data about whether younger or older women cope better. Participants were 155 women (97% Caucasian) recruited within 4-6 weeks after diagnosis (Stage I n = 81; Stage II n = 56; Stage III n =18). Age ranged from 30-84 (M = 55). At baseline and again 5-6 months later, we related age to mood (the Profile of Mood States), quality of life (QOL; measured with the FACT-B), and avoidant coping (from the Coping Response Indices). At baseline, older women consistently reported better moods (rs ranging from -.23 for fatigue to -.37 for anger; all ps < .05). Older women also reported a better QOL, with correlations ranging from .20 (physical well being) to .33 (emotional well being). Mood and quality of life both improved significantly over time; more importantly, the strength of the age/mood and age/QOL relationships declined at the follow-up. Age was only related to anger (r = –.28), depression (r = –.16), and emotional well being (r = .22). Age was unrelated to avoidant coping at either interval (rs < -.12). Overall, these data suggest that age/mood relationships depend on the measurement interval, and we speculate about differences between younger and older women (e.g., child rearing responsibilities) that may contribute to greater distress at the time of diagnosis.

Supported by NIH Grant # RO1 CA77756

CORRESPONDING AUTHOR: Kevin D. McCaul, Ph.D., Department of Psychology, North Dakota State University, Fargo, ND 58105 USA

E-87

HOW DO BREAST CANCER SURVIVORS FEEL ABOUT RECURRENCE RISK?

Kevin D. McCaul, Ph.D., Shannon C. Erickson*, B.A., Kathleen M. Romanek, B.A., North Dakota State University; and Ann K. Sandgren, Ph.D., Roger Maris Cancer Center

We examined the beliefs that breast cancer survivors have about their risk of breast cancer recurrence. Participants were 155 women (97% Caucasian) recruited within 4-6 weeks of diagnosis (Stage I n = 81; Stage II n = 56; Stage III n =18) and interviewed during treatment and 5-6 months later. On a 1 (no chance) to 5 (guaranteed to happen) scale, women judged the chances that they might redevelop breast cancer again sometime in their life. Scores were stable across time (Ms = 2.50 and 2.41 at baseline and follow-up). Women also compared their risk to other women with breast cancer (1 = much less; 3 = the same; 5 = much greater). Overall, they perceived their risk to be smaller than others' (Ms = 2.50 and 2.39 at baseline and follow-up). Cancer stage failed to predict judgments of absolute risk, weakly predicted comparative risk (r = .20 at baseline, p < .05), and was unrelated to cancer worries (e.g., about recurrence or dying). Averaged across risk measures, greater perceived risk predicted (ps < .05) greater worry about recurrence at baseline (r = .37) and follow-up (r = .25). Greater risk also predicted more intrusive thoughts at baseline (r = .23) but not at follow-up. Survivor worries about recurrence may be more dependent on their subjective risk perceptions than on more objective predictive variables such as staging. We need to examine how health professionals communicate information about recurrence risk to breast cancer survivors.

Supported by NIH Grant # RO1 CA77756

CORRESPONDING AUTHOR: Kevin D. McCaul, Ph.D., Department of Psychology, North Dakota State University, Fargo, ND 58105

E-88

THE IMPACT OF SPIRITUALITY ON DESIRE FOR HASTENED DEATH, HOPELESSNESS, AND QUALITY OF LIFE IN TERMINALLY ILL CANCER PATIENTS

Colleen S. McClain, B.A.* and Barry Rosenfeld, Ph.D., Fordham University; and William Breitbart, M.D., Memorial Sloane-Kettering Cancer Center

Living with a terminal illness need not be wrought with intense psychological suffering. Spirituality may offer the serenity often lacking in such times. The goal of this study was to ascertain the relationship between spiritual well-being and end-of-life despair among a group of terminally ill cancer patients. The sample consisted of 186 hospice patients with life expectancies of less than 6 months. Demographics included a mean age of 65.05 years, 56% female, 73.7% Caucasian, 19.4% African American, 5.9% Hispanic. The primary independent variable was the FACIT Spiritual Well-Being Scale. The dependent variables included the Schedule of Attitudes toward Hastened Death, Beck Hopelessness Scale, and an abbreviated version of the McGill Quality of Life Questionnaire. Results demonstrated significant negative correlations (p<.001) between spiritual well-being and desire for hastened death and hopelessness and significant positive correlations (p<.001) between spiritual well-being and quality of life. Multiple regression analyses indicated that spiritual well-being level is the strongest predictor of the outcome variables: desire for death (beta=–.46, p<.001), hopelessness (beta=–.64, p<.001), and quality of life (beta=.31, p=.001), contributing over and above measures of depression and other relevant variables. Further regression analyses indicated a significant interaction between spirituality and depression in predicting desire for hastened death (beta=–.41, p=.002), demonstrating that spiritual well-being acts as a moderating variable. Implications for clinical interventions, such as providing more spiritual assessment and guidance/exploration, are discussed.

This research was supported by a grant from the National Institute for Nursing Research (NR 05183, W. Breitbart, P.I.) and a grant from the American Foundation of Suicide Prevention (B. Rosenfeld, P.I.).

CORRESPONDING AUTHOR: Colleen McClain, 215 E. 95th Street, 19G, New York, NY 10128

E-89

ENHANCING ADAPTATION TO PROSTATE CANCER RISK FEEDBACK AND SCREENING ADHERENCE.

Suzanne M. Miller*, Kerry Sherman, Michelle Rodoletz, Lisa Brower, Michael Diefenbach, Laura Stanton and James Babb, Fox Chase Cancer Center

The utility of a theory-based counseling intervention for enhancing adaptation to prostate cancer screening feedback among at-risk men was assessed. Participants (N=62; mean age 48; 63% Caucasian; 71% married; 52% college degree) received a standard education session and were randomized into either a Cognitive Affective Preparation (CAP) intervention promoting cognitive-affective processing of, and preparation for, risk feedback and its consequences or a General Health Information (GHI) control. Knowledge, risk perceptions, risk-related distress and intentions to adhere were assessed at baseline (risk-assessment program entry), 1-week, and 6-months post-screening. Additionally, adherence to the 1-year follow-up screening visit was assessed. Upon entry, participants were further categorized into high monitors (who attend to health threats) and low monitors (who distract from health threats). Although knowledge was generally high, many participants (71%) answered 25% of baseline knowledge questions incorrectly. Knowledge levels increased post-screening (p<.05), especially for married men (p<.05). Participants consistently underestimated their prostate cancer risk, with 44% considering themselves at average or below average risk even at the 6-month follow-up. Analysis of variance (controlling for relevant confounders) found that high monitors receiving CAP manifested less risk-related distress 1-week post-screening feedback (p<.05) and, over time, greater intentions to adhere to follow-up recommendations (p<.01). Conversely, low monitors displayed less risk-related distress and greater intentions to adhere when receiving GHI. A logistic regression found that higher levels of monitoring (p=.03; Wald c2=4.56) and moderate levels of post-screening distress (p=.05;Wald c2=3.79) predicted actual follow-up adherence. Findings indicate the importance of tailoring health communications to individual attentional style to facilitate adjustment and adherence.

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111

C-103

THE RELATIONSHIP BETWEEN PROACTIVITY AND DISEASE PROGRESSION IN ALS PATIENTS

Erika J. Wolf, Harvard University

The purpose of this research was to investigate the relationship between proactive behaviors and disease progression in 2 studies of Amyotrophic Lateral Sclerosis (ALS) patients. Subjects in study 1 (n = 72, 67% male, mean age = 53, 100% White) answered the Proactivity Scale (a self-report, 3 subscale inventory of active coping behaviors specific to the ALS population) and the Revised ALS Functional Rating Scale (ALSFRS-R; Cedarbaum et al., 1999), a self-report measure of muscle function. Subjects were grouped according to muscle function and diagnosis date. Four 2 X 4 ANOVAs with planned contrasts suggest that patients in different stages of the disease are proactive in different ways. Those subjects with poor muscle function reported seeking medical information about ALS significantly more than those with better muscle function ($p < .05$). Subjects with poor muscle function and older diagnosis dates reported seeking and offering social support within the ALS community significantly more than other groups ($p < .05$). Subjects with more muscle function were significantly more likely to report taking part in mainstream leisure activities despite ALS ($p < .05$). Seven months after study 1, 28 subjects participated in study 2 by answering the ALSFRS-R again. T-tests suggest that subjects with high study 1 proactivity scores (in terms of seeking and offering social support in the ALS community) had less loss of muscle function by the time of study 2 than subjects low in this measure of proactivity ($p < .05$). Key conclusions to be discussed include the importance of population-specific coping inventories, the interplay of disease stage and coping behaviors, and the importance of ALS community social support to later muscle function.

CORRESPONDING AUTHOR: Erika J. Wolf, VA Boston Healthcare System (116B2), 150 South Huntington Ave., Boston, MA 02130

C-104

TOWARD AN EMPIRICALLY-BASED MEASURE OF SOCIAL SUPPORT: THE AESSI

Sheila Alford, B.A., Leslie Shaw, B.S., Elwood Robinson, Ph.D., North Carolina Central University; Laura E. Dreer, M.A., Patrick Logue, Ph.D., and Christopher L. Edwards, Ph.D., Duke University Medical Center

In recent years, social support has been found to influence several medical and psychological outcomes in chronic pain management, oncology, hematology, and many others areas. Interestingly, very few theoretically-based, well-validated, and psychometrically sound measures are available to objectively quantify social support. The Alford-Edwards Social Support Inventory (AESSI) was developed in response to that need using the empirical literature, focus groups, and consultation with mental health professionals with expertise in social support. The AESSI is used to assess satisfaction with 4 theory-driven components of social support (Emotional, Instrumental, Informational, Comparison) from 5 primary sources (Spouse/ Significant Other, Friends, Family, Co-workers, Other Social Contacts).

As part of a larger IRB-approved investigation of the factors that predict postpartum psychopathology in low-income women, the AESSI was administered to 13 female participants and raw scores were correlated with the Symptoms Checklist 90-item Revised (SCL-90-R) and the Edinburgh Postnatal Depression Scale (EPDS) to assess the measure's initial psychometric properties.

The preliminary results indicated that the total score on the AESSI correlated significantly with the Comparison ($p=.0002$), Instrumental, Informational, and Emotional support subscales ($p=.0001$) as well as the expressed degree of satisfaction with social support from Friends ($p=.02$) and Family (.007). The AESSI total score further correlated with the Anxiety ($p=.05$), Hostility ($p=.008$), and Paranoia ($p=.01$) scales of the SCL-90-R with trends toward significance on the Phobic Anxiety ($p=.09$), Interpersonal Sensitivity ($p=.09$), and Depression scales (.07). The psychometric properties of the AESSI will be described as subsequent versions and population-specific norms are developed.

CORRESPONDING AUTHOR: Christopher L. Edwards, Duke University Medical Center, 932 Morreene Rd., Rm. 166, Durham, NC 27710

C-105

PSYCHOSOCIAL PREDICTORS OF PREGNANCY OUTCOME AMONG COUPLES UNDERGOING IN-VITRO FERTILIZATION

Kerry A. Sherman*1, Ph.D., Miranda Montrone2, M.A., Suzanne M. Miller1, Ph.D., John Tyler2, M.D., and Geoff Driscoll2, M.D.

1Fox Chase Cancer Center, Philadelphia and 2City West I.V.F., Sydney, Australia

This study explored the role of coping responses and attentional style on pregnancy outcome following In-Vitro-Fertilization (IVF) treatment among Australian couples. Both members of medically diagnosed infertile couples (N = 81; female: 87% Caucasian, mean age 33; male: 94% Caucasian, mean age 34) provided demographic information and completed the Brief COPE and the Monitor Blunter Style Scale prior to commencing their first IVF treatment cycle at an Australian clinic. Pregnancy outcome was determined through the clinic medical records for each couple six months following commencement of treatment. Forty-seven percent of couples had become pregnant. Chi-squared analyses indicated that among women, humor as a coping strategy ($p<.05$), and among men, lower intrusive ideation ($p<.05$), were associated with higher pregnancy rates. Further, low monitoring women (who distract from threat-related cues) utilizing humor ($p<.05$) were more likely to become pregnant, versus those not utilizing humor. Logistic regression analyses were computed to determine the best predictors of pregnancy outcome. Use of humor ($p<.05$) for women as a coping response was the only significant predictor of pregnancy, with a trend for low monitoring attentional style being predictive of pregnancy. Lower levels of intrusive ideation in men was also a significant predictor of pregnancy ($p<.01$). Results suggest that coping responses of both members of a couple are important factors influencing pregnancy outcome in IVF treatment for women. Therefore, the attentional style and coping responses of couples need to be considered when designing infertility treatment counseling programs.

CORRESPONDING AUTHOR: Kerry A. Sherman, Ph.D., Department of Behavioral Medicine, Fox Chase Cancer Center, 7711 Burholme Avenue, Philadelphia, PA 19111 USA

C-106

HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND PSYCHIATRIC SYMPTOMS IN MEN: CORTISOL, DHEA, AND THE CORTISOL/DEHYDROEPIANDROSTERONE RATIO

Elizabeth A. Shirtcliff, Douglas A. Granger, and Alan Booth, The Pennsylvania State University

I.Objective. Clinical, behavioral, and experimental findings suggest that individual differences in the activity of the hypothalamic-pituitary-adrenal axis (i.e., cortisol) are linked to abnormal cognitive and emotional problems associated with depression and anxiety. We evaluate the nature and magnitude of the effects of DHEA(s), an anti-glucocorticoid produced by the HPA axis, on these symptoms modeling relationships with the cortisol/DHEA(s) ratio. II. Method. We investigated this effect in a large random sample (n = 4,378) of men ages 30 to 48 who received a psychiatric interview, medical exam, and had AM blood samples assayed for cortisol and DHEA(s). III. Results. Analysis revealed curvilinear associations between the cortisol/DHEA(s) ratio and symptoms of phobia, panic, depression, and antisocial behavior, with risk of symptoms increasing only when cortisol levels were very high relative to DHEA(s) levels. The relationship between the cortisol/DHEA(s) ratio and symptomatology was independent of current health status, unemployment, marital status, and body mass. On the other hand, there was no evidence that higher DHEA levels alone, or higher DHEA levels relative to cortisol levels associated with low levels of psychiatric symptoms IV. Conclusion. Our knowledge about relationships between the HPA axis activation and abnormal cognitive and emotional processes may have been obscured by the almost exclusive reliance on cortisol to operationalize the dynamic, integrated, and synergistic nature of the HPA axis in prior studies. Our observations also suggest that blanket self-prescription of DHEA may not improve psychological well-being in the majority of individuals. Supplementation might have the most beneficial effects when cortisol levels are very high relative to endogenous levels of DHEA.

CORRESPONDING AUTHOR: Elizabeth A. Shirtcliff, Department of Biobehavioral Health, 315 Health and Human Development East, The Pennsylvania State University, University Park, PA 16802; Tel: 814-865-8442; Fax: 814-863-7525; Email: bshirtcliff@psu.edu

ATTENTIONAL STYLE AND ADJUSTMENT TO PARTICIPATION IN GENETIC TESTING FOR INHERITED BREAST AND OVARIAN CANCER RISK

Miller, S.M.*, Driscoll, J.L, Rodoletz, M., Sherman, K.A., Buzaglo, J.S., Daly, M., Godwin, A., & Babb, J.
Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA, USA

Guided by the Cognitive-Social Health Information Processing (C-SHIP) model, this study focused on the psychosocial factors involved in decision-making for *BRCA* genetic testing among women (N=281) undergoing genetic counseling because of a putative hereditary family history of breast and/or ovarian cancer. We explored whether high monitors (who scan for and amplify disease-related threats) differed in their cognitive and affective reactions to testing in comparison with low monitors (who typically distract from threat-related cues). Participants attended an initial group breast and ovarian cancer educational session, followed by an individualized cancer-risk-counseling session. Psychosocial assessments were completed prior to each session and one week following the decision to donate blood for genetic testing. Outcomes included satisfaction with the decision to donate blood and disease-related intrusive and avoidant ideation, as measured by the Revised Impact of Events Scale (RIES). The majority of the sample (60%) were unaffected relatives and well-educated, with 67% having at least a college education. The average age was 47 years (SD=12). There were no significant differences in the decision to donate blood based on attentional style, with 95% providing a blood sample. However, high monitors were more satisfied with their decision to donate blood one-week post-blood draw than were low monitors ($p \leq .05$). Nonetheless, high monitors displayed greater disease-specific intrusive ($p \leq .05$) and avoidant ($p \leq .01$) ideation than low monitors did. These results suggest that it is important to design counseling and outreach educational programs that are tailored to the individual's coping style. Ongoing research seeks to extend these findings to a sample of low income, minority women.

The 26th Meeting of the American Society of Preventive Oncology

THE ROLE OF MONITORING AND ANTICIPATED *BRCA1/2* CARRIER STATUS ON FAMILY COMMUNICATION AMONG WOMEN WITH A HEREDITARY PATTERN.

Suzanne M. Miller, Kerry Sherman, Michelle Rodoletz, Joanne Buzaglo, Jennifer Driscoll, Mary Daly, Andrew Godwin, & James Babb, Fox Chase Cancer Center

This study explored the role of anticipated carrier status and attentional style on intentions and plans for communicating *BRCA1/2* test results among women with a putative hereditary pattern of breast/ovarian cancer. Women (N=196; 95% Caucasian; mean age 49; 59% unaffected with breast/ovarian cancer) provided demographic information and completed the Monitor-Blunter Style Scale prior to receiving cancer risk counseling (baseline). Information was also obtained on intentions to communicate risk feedback to family members at baseline and one-week following the donation of blood for genetic testing. At baseline, most participants were certain they would communicate results to their family (71%) and children (66%), independent of attentional style and potential outcome. Participants were more inclined to seek advice about ($p<.01$), and carefully plan for ($p<.01$), communication of positive (versus negative) test results. However, even when anticipating positive results, only 41% reported serious intentions to seek advice about or carefully plan their communication. Analysis of variance indicated that low monitors (who distract from threat-related cues) reported significantly less intentions to communicate positive test results to family members ($p<.01$), and were less likely to engage in strategic planning ($p<.05$) one-week post-blood draw than high monitors (who scan for disease-related threats). Results suggest that women are motivated to share their carrier status with their family, particularly in the case of a positive result, but do not seek advice or strategically prepare for disclosure of test feedback. Attentional style should be considered when designing genetic testing counseling programs.

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA, 19111

The 26th Annual Meeting of the American Society of Preventive Oncology

PROSTATE CANCER RISK ASSESSMENT: ENHANCING PARTICIPATION AND ADAPTATION

Miller, S.M., Sherman, K., Rodoletz, M., Brower, L., Diefenbach, M., & Bruner, D.

The utility of a theory-based intervention to enhance on-going participation in, and adaptation to, a Prostate Cancer Risk Assessment Program (PRAP) was assessed. High-risk men (N=62; M age 48; 63% Caucasian; 71% married; 52% college degree) received education and either Cognitive-Affective Preparation (CAP), to promote in-depth processing of, and preparation for, risk feedback/disease management, or a General Health Information (GHI) control. Participants were categorized into *high monitors* (attend to health threats) and *low monitors* (distract from health threats). The majority of participants (71%) answered 25% of baseline knowledge questions incorrectly. Knowledge improved post-screening, especially for married men ($p<.05$). Men consistently underestimated their prostate cancer risk, with 44% believing they were below average to average risk, even at 6-months. High monitors receiving CAP had less distress 1-week post-feedback ($p<.05$) and, over time, greater intentions to adhere ($p<.01$) than high monitors receiving the GHI. Conversely, low monitors had less distress and greater intentions when receiving GHI versus CAP. High monitoring ($p=.03$) and moderate post-feedback distress ($p=.05$) predicted actual adherence at 1-year follow-up. Tailored communications facilitate adaptation to risk feedback and adherence.

200 words

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., 7701 Burholme Ave., Philadelphia, PA, 19111

The 26th meeting of the American Society of Preventive Oncology

THE ROLE OF MONITORING AND ANTICIPATED *BRCA1/2* CARRIER STATUS ON FAMILY COMMUNICATION INTENTIONS AND PLANS AMONG WOMEN WITH A HEREDITARY PATTERN FOR BREAST/OVARIAN CANCER.

Suzanne M. Miller, Kerry Sherman, Michelle Rodoletz, Joanne Buzaglo, Jennifer Driscoll, Mary Daly, Andrew Godwin, & James Babb, Fox Chase Cancer Center

This study explored the role of anticipated carrier status and attentional style on intentions and plans for communicating *BRCA1/2* test results among women with a putative hereditary pattern of breast/ovarian cancer. Women (N=196; 95% Caucasian; mean age 49; 59% unaffected with breast/ovarian cancer) provided demographic information and completed the Monitor-Blunter Style Scale prior to receiving cancer risk counseling (baseline). Information was also obtained on intentions to communicate risk feedback to family members at baseline and one-week following the donation of blood for genetic testing. At baseline, most participants were certain they would communicate results to their family (71%) and children (66%), independent of attentional style and potential outcome. Participants were more inclined to seek advice about ($p<.01$), and carefully plan for ($p<.01$), communication of positive (versus negative) test results. However, even when anticipating positive results, only 41% reported serious intentions to seek advice about or carefully plan their communication. Analysis of variance indicated that low monitors (who distract from threat-related cues) reported significantly less intentions to communicate positive test results to family members ($p<.01$), and were less likely to engage in strategic planning ($p<.05$) one-week post-blood draw than high monitors (who scan for disease-related threats). Results suggest that women are motivated to share their carrier status with their family, particularly in the case of a positive result, but do not seek advice or strategically prepare for disclosure of test feedback. Attentional style should be considered when designing genetic testing counseling programs.

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA, 19111

The 60th Annual Meeting of the American Psychosomatic Society

ENHANCING ADAPTATION TO AND PARTICIPATION IN PROSTATE CANCER RISK PROGRAMS

Suzanne M. Miller, Kerry Sherman, Michelle Rodoletz, Lisa Brower, Michael Diefenbach, Laura Stanton, Behavioral Medicine, & James Babb, Biostatistics, Fox Chase Cancer Center, Philadelphia, PA

The utility of a theory-based counseling protocol to enhance adaptation to and ongoing participation in a Prostate Cancer Risk Assessment Program (PRAP) was assessed. Men at increased risk (N=62; M age 48; 63% Caucasian; 71% married; 52% college degree) received PRAP education and either a Cognitive-Affective Preparation (CAP) intervention promoting in-depth processing of, and preparation for, risk feedback and management *or* a General Health Information (GHI) control. Knowledge, risk perceptions, risk-related distress and intentions to adhere were assessed upon entry, and 1-week and 6-months post-feedback. At entry, participants were also categorized into high monitors (who attend to health threats) and low monitors (who distract from health threats). Additionally, adherence to 1-year follow-up screening was assessed. The majority of men (71%) answered 25% of baseline knowledge questions incorrectly. Knowledge levels increased post-screening ($p<.05$), especially for married men ($p<.05$). Prostate cancer risk was consistently underestimated, with 44% endorsing an average or below average risk even at 6-month follow-up. An ANOVA found high monitors receiving CAP had less distress 1-week post-feedback ($p<.05$) and, over time, greater intentions to adhere to follow-up ($p<.01$). Conversely, low monitors had less distress and greater intentions to adhere when receiving GHI. Logistic regression revealed high levels of monitoring ($p=.03$) and moderate levels of post-feedback distress ($p=.05$) predicted adherence. Tailoring communications to attentional style may facilitate adaptation to prostate-cancer risk and program adherence.

CORRESPONDING AUTHOR: Suzanne M. Miller, Ph.D., FCCC, 7701 Burholme Ave., Philadelphia, PA, 19111

The 60th Meeting of the American Psychosomatic Society

COPING STRATEGIES AND PREGNANCY OUTCOME AMONG COUPLES UNDERGOING IN-VITRO FERTILIZATION

Kerry A. Sherman*[1], Ph.D., Miranda Montrone[2], M.A., John Tyler[2], M.D., Geoff Driscoll[2], M.D., & Suzanne M. Miller[1], Ph.D.
[1]Fox Chase Cancer Center, Philadelphia, U.S.A.
[2]City West I.V.F., Sydney, AUSTRALIA.

This study explored the role of coping responses and attentional style on pregnancy outcome following In-Vitro-Fertilization (IVF) treatment among Australian couples. Both members of medically diagnosed infertile couples (N = 81; female: 87% Caucasian, mean age 33; male: 94% Caucasian, mean age 34) provided demographic information and completed the Brief COPE and the Monitor Blunter Style Scale prior to commencing their first IVF treatment cycle at an Australian clinic. Pregnancy outcome was determined through the clinic medical records for each couple six months following commencement of treatment. Forty-seven percent of couples had become pregnant. Chi-squared analyses indicated that among women, humor as a coping strategy ($p<.05$), and among men, greater intrusive ideation ($p<.05$), were associated with higher pregnancy rates. Further, low monitoring women (who distract from threat-related cues) utilizing humor ($p<.05$) were more likely to become pregnant, versus those not utilizing humor. Logistic regression analyses were computed to determine the best predictors of pregnancy outcome. Use of humor ($p<.05$) for women as a coping response was the only significant predictor of pregnancy, with a trend for low monitoring attentional style being predictive of pregnancy. Higher levels of intrusive ideation in men was also a significant predictor of pregnancy ($p<.01$). Results suggest that coping responses of both members of a couple are important factors influencing pregnancy outcome in IVF treatment for women. Therefore, the attentional style and individual coping responses of couples need to be considered when designing infertility treatment counseling programs.

CORRESPONDING AUTHOR: Kerry A. Sherman, Ph.D., Department of Behavioral Medicine, Fox Chase Cancer Center, 7711 Burholme Avenue, Philadelphia, PA 19111, USA

60th Annual Meeting of the American Psychosomatic Society

ENHANCING PARTICIPATION IN AND ADAPTATION TO PROSTATE CANCER RISK ASSESSMENT PROGRAMS AND SCREENING ADHERENCE

SM Miller, KA Sherman, M Rodoletz, L Brower, MA Diefenbach, L Stanton, D Bruner

Purpose: The utility of a theory-based counseling intervention for improving ongoing participation in prostate cancer risk assessment programs and adaptation to screening feedback among at-risk men was assessed. Methods: Participants (N=62; mean age 48; 63% Caucasian; 71% married; 52% college degree) received a standard education session and were randomized into either a Cognitive Affective Preparation (CAP) intervention promoting cognitive-affective processing of, and preparation for, risk feedback and its consequences or a General Health Information (GHI) control. Knowledge, risk perceptions, risk-related distress and intentions to adhere were assessed at baseline (risk-assessment program entry), 1-week, and 6-months post-screening. Additionally, adherence to the 1-year follow-up screening visit was assessed. Upon entry, participants were further categorized into *high monitors* (who attend to health threats) and *low monitors* (who distract from health threats). Results: Although knowledge was generally high, the majority of participants (71%) answered 25% of baseline knowledge questions incorrectly. Knowledge levels increased post-screening ($p<.05$), especially for married men ($p<.05$). Participants consistently underestimated their prostate cancer risk, with 44% considering themselves at average or below average risk, even at the 6-month follow-up. Analysis of variance (controlling for relevant confounders) found that *high monitors* receiving CAP manifested less risk-related distress 1-week post-screening feedback ($p<.05$) and, over time, greater intentions to adhere to follow-up recommendations ($p<.01$). Conversely, *low monitors* displayed less risk-related distress and greater intentions to adhere when receiving GHI. A logistic regression found that higher levels of monitoring ($p=.03$) and moderate levels of post-screening distress ($p=.05$) predicted actual follow-up adherence. Conclusions: Findings indicate tailoring health communications to individual attentional style may facilitate psychological adjustment to prostate cancer risk assessment and adherence to recommended screening.

The 2nd Annual Meeting of the American Academy of Health Behavior

PSYCHOSOCIAL PREDICTORS OF PREGNANCY OUTCOME AMONG COUPLES UNDERGOING IN-VITRO FERTILIZATION

KA Sherman, M Montrone, J Tyler, G Driscoll, SM. Miller, J. Knowles.

Purpose: This study explored the role of coping responses and attentional style on pregnancy outcome following In-Vitro-Fertilization (IVF) treatment among Australian couples. Methods: Both members of medically diagnosed infertile couples (N = 81; female: 87% Caucasian, mean age 33; male: 94% Caucasian, mean age 34) provided demographic information and completed the Brief COPE and the Monitor Blunter Style Scale prior to commencing their first IVF treatment cycle at an Australian clinic. Pregnancy outcome was determined through the clinic medical records for each couple six months following commencement of treatment. Results: Forty-seven percent of couples had become pregnant. Chi-squared analyses indicated that among women, humor as a coping strategy ($p<.05$), and among men, higher intrusive ideation ($p<.05$), were associated with higher pregnancy rates. Further, low monitoring women (who distract from threat-related cues) utilizing humor ($p<.05$) were more likely to become pregnant, versus those not utilizing humor. Logistic regression analyses were computed to determine the best predictors of pregnancy outcome. Use of humor ($p<.05$) for women as a coping response was the only significant predictor of pregnancy, with a trend for low monitoring attentional style being predictive of pregnancy. Greater levels of intrusive ideation in men was also a significant predictor of pregnancy ($p<.01$). Conclusions: Results suggest that coping responses of both members of a couple are important factors influencing pregnancy outcome in IVF treatment for women. Therefore, the attentional style and coping responses of couples need to be considered when designing infertility treatment counseling programs.

CORRESPONDING AUTHOR: KA. Sherman

2nd Annual Meeting of the American Academy of Health Behavior

Gli stili comportamentali di *monitoring* e *blunting* nella gestione delle minacce alla salute: l'esempio del rischio e della malattia oncologica

Suzanne M. Miller, Kerry A. Sherman,
Joanne S. Buzaglo, Michelle Rodoletz
Fox Chase Cancer Center[*]

Riassunto. Nel corso dell'ultimo decennio la ricerca nell'ambito della psicologia della salute ha utilizzato con successo concetti psicologici basilari nell'intento di ampliare l'attuale comprensione del ruolo delle differenze individuali nella gestione di sfide e stressor connessi allo stato di salute (es. Lazarus, 1991; Rothman e Salovey, 1997; Taylor, 1990). In questo lavoro applicheremo alla psicologia della salute una cornice teorica composita, ovvero l'approccio "cognitivo-affettivo" alle differenze individuali (Miller *et al.*, 1996d), che integra una serie di dati e riflessioni teoriche provenienti da diverse aree delle scienze cognitivo-comportamentali e dalla valutazione di interventi di consulenza e comunicazione (es. Carver e Scheier, 1981; Sarason, 1979). Iniziamo con una panoramica su questa cornice cognitivo-affettiva, che delinea il processo attraverso il quale gli individui elaborano cognitivamente e affettivamente le informazioni riguardanti minacce alla propria salute (Miller e Schnoll, 2000). In particolare, vogliamo evidenziare due stili caratteristici ("*monitoring*" e "*blunting*"[1]) di risposta alle minacce alla salute, utilizzando come esempio il contesto oncologico. Il cancro offre un paradigma ideale per lo studio delle risposte a sfide connesse alla salute, dal momento che le minacce legate al cancro sono spesso realistiche e complesse; richiedono dunque la capacità continuativa di prendere decisioni, l'aderenza e la modulazione dell'ansia nel tempo (Miller e Diefenbach, 1998a). Inoltre, il contesto oncologico tocca un'ampia gamma di situazioni, da individui sani con rischio medio di cancro, a individui con alto rischio (ad esempio per predisposizione genetica, o per una precedente esposizione a elementi cancerogeni, o per svantaggio socio-demografico), a individui ai quali è stato già diagnosticato il cancro, a individui che sono sopravvissuti al cancro.

Summary. Over the past decade, health psychology research has successfully applied basic psychological concepts to furthering our understanding of the role of individual differences in dealing with heath-related challenges and stressors (e.g., Lazarus, 1991; Rothman & Salovey, 1997; Taylor, 1990). In this paper, we will apply to health psychology a comprehensive theoretical framework, the "cognitive-affective" approach to individual differences (Miller, Shoda & Hurley, 1996d), that integrates cumulative findings and theorizing from diverse areas of cognitive-behavioral science and evidence-based counseling and communication interventions (e.g., Carver & Scheier, 1981; Sarason, 1979). First, we provide an overview of our cognitive-affective framework, which delineates how individuals cognitively and affectively process information about health threats (Miller & Schnoll, 2000). In particular, we highlight two distinctive cognitive-affective signatures ("*monitoring*" versus "*blunting*") in response to health

[*] La corrispondenza va indirizzata a Suzanne M. Miller, Ph.D., Division of Population Science, Psychosocial and Behavioral Medicine Program, Fox Chase Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania, 19111, Usa.

[1] La traduzione letterale di questi due termini è la seguente: controllante e smussante.

threats, using the cancer context as an exemplar. Cancer provides an ideal paradigm for studying the response to health-related challenges, since cancer-related threats are often probabilistic and complex. Hence, they require sustained decision making, adherence, and modulation of anxiety over time (Miller & Diefenbach, 1998a). Further, cancer spans the spectrum of healthy individuals with average cancer risk, to individuals with high risk (e.g., due to genetic predisposition, prior exposure to carcinogens, or socio-demographic disadvantage), to individuals already diagnosed with cancer, to cancer survivors.

L'elaborazione cognitivo-affettiva delle informazioni riguardanti il rischio o la malattia oncologica

Il modello dell'Elaborazione Cognitivo-Sociale di Informazioni sulla Salute (Cognitive-Social Health Information Processing, C-SHIP), di recente sviluppo, offre un'ampia cornice che in sé racchiude i basilari processi cognitivo-affettivi sottostanti al modo in cui gli individui codificano, percepiscono e reagiscono, a livello comportamentale ed emotivo, a informazioni potenzialmente stressanti sul proprio stato di salute (Miller *et al.*, 1996d; Miller *et al.*, in press). Questo approccio evidenzia cinque unità principali di mediazione cognitivo-affettiva (C-SHIP) che vengono attivate ogni qual volta si affrontano minacce alla propria salute e che determinano il comportamento adattivo non solo a breve termine, ma anche nel tempo (Leventhal *et al.*, 1992; Miller *et al.*, 1996d). Queste unità di mediazione includono le codifiche e le inferenze dell'individuo, le sue aspettative e convinzioni riguardanti il cancro, i suoi sentimenti ed emozioni, i suoi valori e obiettivi per la salute, e le sue strategie di coping e autoregolazione (Miller *et al.*, 1996d). Nella prospettiva del modello C-SHIP, gli individui si differenziano, nelle risposte prototipiche o caratteristiche a informazioni sulla salute, fondamentalmente per due aspetti: la facilità di attivazione delle unità di mediazione e la struttura della relazione tra le unità di mediazione una volta attivate (Miller *et al.*, 1996d). Ad esempio, per quanto riguarda la facilità di attivazione, venire a sapere che è stato diagnosticato un cancro al seno a una parente stretta può aumentare la vulnerabilità percepita di una donna mentre, per un'altra donna, tale informazione può non avere alcun effetto. In termini di pattern di attivazione tra le unità di mediazione, la vulnerabilità percepita può accendere l'aspettativa di una donna di sviluppare un cancro al seno e il convincimento che non ci sia niente che ella possa fare per diminuire il rischio di un cancro, e quindi aumentare la sua angoscia. Viceversa, un'altra donna che si senta ugualmente vulnerabile alla malattia può credere che, sottoponendosi agli esami raccomandati, potrà individuare la malattia a uno stadio precoce e di conseguenza ciò ridurrà la sua angoscia.

I tratti comportamentali del *monitor* e del *blunter* in risposta a minacce alla salute

Nella nostra ricerca ci siamo concentrati su due stili chiave prototipici o caratteristici di elaborazione delle informazioni connesse a minacce alla salute: il "*monitoring*" e il "*blunting*" (Miller e Diefenbach, 1998a). I *monitor* generalmente cercano ed esaltano segnali minacciosi riguardanti la salute, mentre i *blunter* distolgono lo sguardo e minimizzano le informazioni minacciose (Miller, 1996a). Le conseguenze cognitivo-affettive e comportamentali di questi stili caratteristici sono state valutate in tutta una serie di contesti di rischio per la salute; in particolare in risposta alla comunicazione, prevenzione e gestione del richio di cancro. Si è osservato che questi stili prototipici sono in grado di predire differenze individuali nelle risposte cognitivo-affettive a stressor connessi allo stato di salute e nelle strategie di coping. È stata sviluppata e validata la Monitor-Blunter Style Scale (MBSS) per distinguere tra *monitor* e *blunter* (Miller, 1987). Tale scala è stata tradotta in diverse lingue e utilizzata in vari contesti oncologici, con popolazioni quali pazienti ginecologiche con rischio di cancro all'utero (es. Miller *et al.*, 1999a; Miller *et al.*, 1994), donne con rischio di cancro al seno o alle ovaie (es. Basen-Engquist, 1997; Diefenbach *et al.*, 1999; Lerman *et al.*, 1994a), pazienti con cancro (es. Gard *et al.*, 1988; Lerman *et al.*, 1990), e donne sane che si sottopongono a controlli regolari (Jacob *et al.*, 1992). La scala è stata utilizzata anche in altri contesti di minaccia medica e in altre popolazioni (es. Litt *et al.*, 1995; Miller *et al.*, 1996b; Phipps e Srivastava, 1997; Steptoe e O'Sullivan, 1986). Faremo ora una rassegna sistematica della letteratura riguardante gli stili *monitoring* e *blunting* rispetto a ciascuna delle cinque unità cognitivo-affettive di mediazione.

Codifiche e inferenze. Quando le persone vengono confrontate con una comunicazione nuova o poco familiare sulla salute, devono adattarsi e assimilare queste informazioni nei loro pre-esistenti schemi sulla salute (Miller *et al.*, in press). Le codifiche si riferiscono alle rappresentazioni mentali che l'individuo ha di sè stesso e delle situazioni di minaccia alla salute (Miller *et al.*, 1996d). Le codifiche e le inferenze riguardanti la salute si riferiscono al modo in cui l'individuo valuta la nuova minaccia e le informazioni rilevanti sulla malattia (es. la comunicazione di rischio genetico, la prognosi della malattia, le raccomandazioni per il trattamento). Ad esempio, quando una donna con una storia personale o familiare di cancro al seno scopre un nodulo al seno, questa esperienza probabilmente attiverà dei ricordi di cancro al seno; viceversa una donna senza tale storia di malattia sarà portata a valutare il nodulo come benigno (Miller *et al.*, 1996d). Le differenze individuali nella codifica di

informazioni influenzano la relazione tra lo stato oggettivo di salute dell'individuo e la sua vulnerabilità percepita alla malattia (Miller *et al.*, 1988; Muris e van Zuuren, 1992). Dal momento che i *monitor* cercano e si focalizzano sui segnali di pericolo è probabile che sviluppino delle codifiche più minacciose, rispetto ai *blunter*, quando si imbattono in situazioni stressanti per la propria salute. Ad esempio, in uno studio sulle parenti di primo grado di pazienti con cancro alle ovaie, le *monitor* percepivano di essere a rischio per lo sviluppo della malattia più di quanto non facessero le *blunter*, a prescindere dai livelli effettivi di rischio (Schwartz *et al.*, 1995; Fang *et al.*, in press). Dunque le loro inferenze sul rischio personale di cancro erano caratterizzate da un'elevata percezione di vulnerabilità (Schwartz *et al.*, 1995), che, in ultima analisi, può minare la messa in atto di comportamenti adattivi di protezione della salute (Kash *et al.*, 1992; Lerman *et al.*, 1993a).

Aspettative e convinzioni. Il modo in cui un evento è percepito e conservato in memoria può generare delle aspettative o delle convinzioni riguardo a ciò che è probabile che succeda (Miller *et al.*, 1999c). Le aspettative comprendono la percezione della propria self-efficacy (ad esempio "io sono in grado di fare regolarmente dei controlli al seno") e l'anticipazione degli effetti di particolari comportamenti (ad esempio, "io posso ridurre la probabilità di un cancro al seno se faccio regolarmente dei controlli al seno") (Bandura, 1986; Lau *et al.*, 1989; Leventhal *et al.*, 1992). Tali aspettative possono avere effetti significativi sui susseguenti comportamenti e sulle susseguenti risposte emotive (Carver *et al.*, 1993; Scheier e Carver, 1985; Stefanek e Wilcox, 1991). La ricerca ha mostrato che i *monitor* e i *blunter* sono caratterizzati da aspettative e convinzioni caratteristiche riguardo al genere di minacce alla salute e al loro esito: i *monitor* hanno, con maggiore probabilità, aspettative negative riguardo la severità e le conseguenze delle minacce alla salute. Ad esempio, in uno studio su donne che facevano un follow-up diagnostico in seguito a un Pap test anormale, le *monitor* percepivano la propria condizione come più grave rispetto alle *blunter* ed erano più pronte a dare la colpa a se stesse per i propri problemi di salute (Miller *et al.*, 1994). I *monitor*, inoltre, credono, generalmente più dei *blunter*, che proveranno angoscia in seguito a minaccia di cancro come, ad esempio, la comunicazione dei risultati di un test genetico per donne a rischio di cancro al seno o alle ovaie (Lerman *et al.*, 1994a). In conclusione, dato che i *monitor* con maggiore probabilità amplificano gli aspetti minacciosi di segnali o esperienze connesse al cancro, questa attenzione alla minaccia può promuovere aspettative negative sulla propria condizione, le sue conseguenze e le convinzioni sulla propria capacità di gestire tale minaccia.

Emozioni. Le persone possono essere molto diverse nelle loro risposte emotive a minacce alla propria salute (Horowitz, 1991; Leventhal *et al.*, 1992), particolarmente riguardo il livello di preoccupazione specifico per la malattia (Lerman *et al.*, 1994b) e l'ideazione intrusiva (Lerman *et al.*, 1993a; Lerman *et al.*, 1991a; Lerman *et al.*, 1991b). Si è osservato che gli stili caratteristici *monitoring-blunting* influenzano la durata e l'intensità dell'angoscia provata dalle persone lungo tutto lo spettro della malattia. Ad esempio, tra le donne con rischio familiare di cancro alle ovaie sottoposte a ecografia, le *monitor* riportavano una angoscia maggiore, in risposta ai falsi positivi, di quanto facessero le *blunter*, sia immediatamente che al follow-up un anno dopo (Wardle, 1995). In uno studio sulle decisioni prese da donne a rischio per cancro alle ovaie, le *monitor* tendevano a sentirsi più vulnerabili al cancro e più angosciate e ansiose riguardo il rischio di cancro di quanto si sentissero le *blunter* (Fang *et al.*, in press). Analogamente, tra pazienti con cancro sottoposte a chemioterapia, le *monitor* provavano più ansia prima del trattamento e riportavano livelli più elevati di depressione durante la chemioterapia (Lerman *et al.*, 1990), nonché nausea e vomito più intensi e più a lungo (Gard *et al.*, 1986), di quanto facessero le *blunter*. I *monitor* riportano anche un disagio più prolungato in risposta a procedure diagnostiche e di trattamento rispetto ai *blunter* (Miller e Mangan, 1983; Miller *et al.*, 1994). Considerati insieme questi dati indicano come i *monitor* siano particolarmente vulnerabili ad alti livelli di angoscia e ideazione intrusiva quando confrontati con minacce alla propria salute. Questo senso accentuato di vulnerabilità e angoscia può portare a ideazione e comportamenti evitanti che possono, a loro volta, minare l'aderenza e un coping effettivo (Kash *et al.*, 1992; Lerman *et al.*, 1993a; Miller *et al.*, 1996c) e interferire con i processi decisionali razionali (Miller *et al.*, 1999b).

Valori e obiettivi. I valori si riferiscono al grado di importanza che le persone assegnano alla propria salute. I valori, a loro volta, danno forma agli obiettivi dell'individuo rispetto alle comunicazioni e alle prescrizioni riguardanti la salute (Miller *et al.*, 1996d). Un valore/obiettivo importante si riferisce al desiderio di informazioni dell'individuo e al livello di coinvolgimento nella cura della propria salute: alcuni pazienti vogliono essere informati del rischio di cancro (es. Croyle e Lerman, 1993; Lerman *et al.*, 1994a) e assumono un ruolo attivo nel prendere decisioni (es. Cassileth *et al.*, 1980), mentre altri pazienti preferiscono rimanere disinformati (es. Jones, 1981) e mantenere un ruolo più passivo (es. Degner e Sloan, 1992). Gli stili *monitoring-blunting* influenzano i valori e gli obiettivi connessi alla salute: mentre i *monitor* desiderano e cercano estese informazioni sul proprio stato di salute, i *blunter* preferiscono ottenere informazioni meno dettagliate (Lerman *et al.*,

1993b; Miller, 1987; Steptoe *et al.*, 1991). Ad esempio, uno studio sul comportamento di ricerca di informazioni delle figlie adulte di donne con diagnosi precoce di cancro al seno ha evidenziato che le *monitor* cercavano più informazioni, da organizzazioni che si occupavano di cancro e da giornali, di quanto facessero le *blunter* (Rees e Bath, 2000). Dato il forte bisogno di informazioni dettagliate, le *monitor* tendono ad essere meno soddisfatte della quantità di informazioni ricevute in un contesto standard (Miller e Mangan, 1983; Steptoe e O'Sullivan, 1986). In ambito oncologico, studi su pazienti a uno stadio precoce di cancro al seno (Lerman *et al.*, 1993b) e su pazienti con cancro metastatico (Steptoe *et al.*, 1991) mostrano come i *monitor* siano meno soddisfatti delle comunicazioni del personale medico rispetto ai *blunter*. I *monitor*, inoltre, pretendono generalmente più dei *blunter*, nel senso che danno grande importanza alla gentilezza e alle rassicurazioni del loro dottore e richiedono più interventi medici, come test diagnostici e prescrizioni (Miller *et al.*, 1988).

Strategie di autoregolazione e di coping. Per sostenere l'aderenza a pratiche di protezione della salute e a regimi di trattamento, in particolare nel tempo, le persone devono essere capaci di programmare efficacemente (ad esempio, sviluppando una strategia per ricordarsi di fare i controlli al seno) e di gestire con successo le interferenze dovute all'angoscia per la propria salute (Carver *et al.*, 1989; Miller *et al.*, 1996b; Miller *et al.*, 1996d). In condizioni di rischio moderato, come negli esami di routine, i *monitor* programmano e aderiscono con più facilità dei *blunter* ai regimi consigliati (ad esempio, mammografia, Pap test e esami colon-rettali) (Christensen *et al.*, 1994; Steptoe e O'Sullivan, 1986). I *blunter*, viceversa, spesso non aderiscono dall'inizio al regime consigliato, dal momento che sottostimano il proprio rischio di cancro. In condizioni di alto rischio, però, (ad esempio, diagnosi di cancro) anche i *monitor* possono non essere aderenti, dal momento che la comunicazione di malattia genera alti livelli di angoscia prolungati nel tempo (Miller *et al.*, 1994; Miller *et al.*, 1996b). Tra le donne che si sottopongono a un follow-up diagnostico, le *monitor* riportano una maggiore ideazione intrusiva, con conseguente ideazione evitante, di quanto facciano le *blunter*; tale angoscia, a sua volta, porta a livelli più alti di negazione e di disimpegno mentale e comportamentale (Miller *et al.*, 1996c). L'incapacità di modulare l'angoscia e l'ideazione intrusiva, accompagnata dall'uso di strategie di coping basate sull'evitamento, può alla fine minare l'aderenza a comportamenti adeguati di protezione della salute e interferire con l'adattamento psicologico (Schwartz *et al.*, 1995).

Implicazioni per la "calibratura" di comunicazioni sulla salute

La ricerca suggerisce che calibrare le comunicazioni relative alla salute sulla base dello stile prototipico di elaborazione delle informazioni dell'individuo può facilitare l'adattamento in tre aree principali connesse alla salute: 1) può migliorare l'adattamento delle persone durante procedure diagnostiche o regimi di trattamento; 2) può promuovere l'aderenza ai regimi consigliati per gli esami, la diagnosi e il trattamento; 3) può facilitare il processo decisionale basato sulle informazioni a disposizione (Miller, 1995). Per quanto riguarda gli interventi studiati per migliorare l'adattamento psicologico a procedure mediche, i *monitor* (che sono più attenti alle minacce) reagiscono meglio quando ricevono informazioni dettagliate e rassicuranti che li preparano a quello che stanno per affrontare; i *blunter*, viceversa, preferiscono informazioni più scarne (es. Miller e Mangan, 1983). Ad esempio, le *monitor* sottoposte a mammografia per un follow-up diagnostico, fatto per valutare delle anomalie emerse in un test precedente, hanno beneficiato, in termini di ansia, depressione e stress, dal ricevere informazioni preparatorie dettagliate riguardanti la consultazione diagnostica a cui andavano incontro (Sherman, 1999). Le *blunter*, viceversa, hanno mostrato un aumento delle emozioni negative quando ricevevano informazioni preparatorie dettagliate.

In un altro studio, i *monitor* che venivano aiutati a rilassarsi e ad aumentare il livello di self-efficacy mostravano un migliore adattamento psicologico e comportamentale a una procedura diagnostica invasiva rispetto ai *monitor* che non ricevevano alcuna preparazione (Gattuso *et al.*, 1992). Alti livelli di informazioni dettagliate e comunicazioni stimolanti la self-efficacy aiutano i *monitor* ad abbassare la propria vulnerabilità percepita e a ridurre preoccupazioni e incertezze e inoltre offrono elementi utili per la programmazione e la gestione dell'ansia. Informazioni troppo dettagliate, viceversa, possono sopraffare i *blunter* costringendoli a elaborare cognitivamente e affettivamente informazioni sulle quali preferirebbero non concentrarsi (Miller, 1995).

Per quanto riguarda gli interventi progettati per promuovere l'aderenza a programmi di cura prescritti, i *monitor* in genere sperimentano delle barriere emozionali (ad esempio, "sono troppo ansioso per sottopormi a Pap test regolari"), mentre i *blunter* in genere sperimentano barriere più in termini di codifiche/aspettative (ad esempio, "non ho alcun sintomo, per cui vuol dire che sto bene"). Di conseguenza, gli interventi di comunicazione sul tema della salute devono essere adattati ai particolari profili cognitivo-emotivi di questi individui. Ad esempio, tra le donne sottoposte a un follow-up diagnostico per un Pap test anomalo, la colposcopia, le *monitor* reagivano meglio quando ricevevano informazioni dettagliate e venivano loro suggerite strategie per gestire lo stress; le *blunter*, viceversa, mostravano una migliore aderenza quando

veniva loro comunicata semplicemente l'importanza degli appuntamenti di follow-up (Miller *et al.*, 2001). In un altro studio su donne sottoposte a colposcopia, le *blunter* dimostravano una maggiore aderenza e conoscenza quando ricevevano un messaggio preparatorio che mirava alle barriere di codifica (ad esempio, che enfatizzava gli svantaggi del non sottoporsi a esami regolari). Al contrario, le *monitor* reagivano meglio quando le stesse informazioni venivano presentate con un tono più neutrale e rassicurante (Miller *et al.*, 1999a). L'esposizione a uno schema di perdita può contribuire ad aumentare l'ideazione intrusiva relativa alla malattia e il senso di vulnerabilità, senza riuscire a promuovere un coping adattivo.

Infine, dal momento che i progressi nella tecnologia medica creano sempre maggiori opzioni mediche per gli individui, studi futuri dovranno valutare i modi di calibrare le comunicazioni così da promuovere una strategia efficace per prendere decisioni, particolarmente in contesti ambigui dove non esistono risposte giuste o sbagliate, ad esempio decisioni quali sottoporsi o meno a esami genetici o a interventi di chirurgia profilattica, quando si è sani. Gli interventi vanno quindi calibrati per aiutare le persone a valutare e a prendere in considerazione il proprio stile di elaborazione delle informazioni prima di prendere una decisione. Per raggiungere questo scopo noi abbiamo sviluppato una procedura di Elaborazione Cognitivo-Affettiva (Cognitive-Affective Processing, CAP), progettata per aiutare le persone a "vivere-prima" nella propria mente una situazione e anticipare le proprie reazioni cognitive ed emotive alle diverse possibilità di cura. Questa modalità di vivere-prima può quindi essere utilizzata come un fondamento più solido sul quale basare le decisioni nel campo della salute e col quale gestire le conseguenze psico-sociale di tali decisioni.

Conclusioni

In sintesi, la cornice cognitivo-affettiva offre una base teorica dalla quale partire per comprendere i processi dinamici coinvolti nell'elaborazione di informazioni riguardanti la salute. Con l'avanzare della tecnologia medica sono sorte nuove sfide che richiedono alle persone di elaborare una grande quantità di informazioni complesse e poco familiari sulla salute e sulla comunicazione del rischio e di gestire livelli alti di incertezza e ambiguità. Ad esempio, la possibilità di sottoporsi a test genetici per valutare la predisposizione a malattie ereditarie pone alle persone molteplici sfide cognitivo-affettive. Tra queste la decisione riguardante il fare o meno l'esame genetico, come gestire la decisione di fare l'esame, cosa fare dei risultati dell'esame, a chi comunicare questi risultati, come modificare il proprio comportamento nel tempo. Ancora, i progressi nel trattamento medico hanno portato a un aumento della sopravvivenza alla malattia e a situazioni di malattia cronica che, a loro volta, hanno generato delle sfide uniche in termini di sorveglianza, trattamento e gestione della malattia. In questo articolo, ci siamo soffermati su due stili prototipici di comportamento che caratterizzano la risposta individuale a minacce alla salute. La ricerca futura dovrà esplorare i profili cognitivo-affettivi dei *monitor* e dei *blunter* in modo più raffinato, particolarmente per quanto riguarda il mantenimento di comportamenti complessi orientati al futuro come quelli necessari per un impegno continuativo di protezione della salute (ad esempio, l'aderenza alla mammografia, l'esercizio fisico) e l'elaborazione di informazioni riguardanti la comunicazione di un rischio attuale. Ciò, a sua volta, migliorerà la nostra capacità di sviluppare e valutare, sulla base della teoria, interventi informativi o di consulenza mirati ad aumentare l'adattamento, a promuovere l'aderenza e a facilitare le decisioni informate attraverso protocolli studiati sulla base degli stili individuali. Infine, la ricerca dovrà confrontare l'impatto di diversi sistemi di comunicazione di queste informazioni calibrate, ovvero di sistemi tradizionali (ad esempio, la consulenza faccia a faccia; la consulenza per telefono; le informazioni scritte) a paragone di nuove tecnologie innovative (ad esempio, CD ROM interattivi; comunicazioni via web).

Riferimenti bibliografici

Bandura A. (1986), *Social foundations of thought and action: A social cognitive theory,* Prentice-Hall, Englewood Cliffs, NJ.

Basen-Engquist K. (1997), Ovarian cancer screening and psychosocial issues: Relevance to clinical practice, *Gynecologic Oncology*, 65: 195-196.

Carver C.S., Pozo C., Harris S.D., Noriega V., Scheier M.F., Robinson D.S., Ketcham A.S., Moffat F.L. Jr., Clark K.C. (1993), How coping mediates the effect of optimism on distress: A study of women with early stage breast cancer, *Journal of Personality and Social Psychology*, 65: 375-390.

Carver C.S., Scheier M. (1981), *Attention and self-regulation: A control theory approach to human behavior,* Springer-Verlag, New York.

Carver C., Scheier M., Weintraub J.K. (1989), Assessing coping strategies: A theoretically based approach, *Journal of Personality and Social Psychology*, 56: 267-283.

Cassileth B.R., Zupkis R.V., Sutton-Smith K., March V. (1980), Information and participation preferences among cancer patients, *Annals of Internal Medicine*, 92: 832-836.

Christensen A.J., Smith T.W., Turner C.W., Cundick K.E. (1994), Patient adherence and adjustment in renal dialysis: A person by treatment interactive approach, *Journal of Behavioral Medicine*, 17: 549-566.

Croyle R., Lerman C. (1993), Interest in genetic testing for colon cancer susceptibility: Cognitive and emotional correlates, *Preventive Medicine*, 22: 284-292.

Degner L.F., Sloan J.A. (1992), Decision making during serious illness: What role do patients really want to play?, *Journal of Clinical Epidemiology*, 45: 941-950.

Diefenbach M.A., Miller S.M., Daly M.B. (1999), Specific worry about breast cancer predicts mammography use in women at risk for breast and ovarian cancer, *Health Psychology*, 18: 532-536.

Fang C.Y., Miller S.M., Daly M., Hurley K. (in press), The influence of attentional style on intentions to undergo prophylactic oophorectomy among FDRs, *Psychology and Health.*

Gard D., Edwards P.W., Harris J., McCormack G. (1988), The sensitizing effects of pretreatment measures on cancer chemotherapy nausea and vomiting, *Journal of Consulting and Clinical Psychology*, 56: 80-84.

Gattuso S.M., Litt M.D., Fitzgerald T.E. (1992), Coping with gastrointestinal endoscopy: Self-efficacy enhancement and coping style, *Journal of Consulting and Clinical Psychology*, 60: 133-139.

Horowitz M. (ed.) (1991), *Person schemas and maladaptive interpersonal patterns*, University of Chicago Press, Chicago.

Jacob T.C., Penn N.E., Kulik T.A., Spieth L.E. (1992), Effects of cognitive style and maintenance strategies on breast self-examination (BSE) practice by African American women, *Journal of Behavioral Medicine*, 15: 586-609.

Kash K., Holland J., Halper M., Miller D. (1992), Psychological distress and surveillance behaviors of women with a family history of breast cancer, *Journal of the National Cancer Institute*, 84: 24-30.

Lau R.R., Bernard T.M., Hartman K.A. (1989), Further explorations of common-sense representations of common illnesses, *Health Psychology*, 8: 195-219.

Lazarus R. (1991), *Emotion and adaptation,* Oxford University Press, New York.

Lerman C., Daly M., Masny A., Balshem A. (1994a), Attitudes about genetic testing for breast-ovarian cancer susceptibility, *Journal of Clinical Oncology*, 12: 843-850.

Lerman C., Daly M., Sands C., Balshem A., Lustbader E., Heggan T., Goldstein L., James J., Engstrom P. (1993a), Mammography adherence and psychological distress among women at risk for breast cancer, *Journal of the National Cancer Institute*, 85: 1074-1080.

Lerman C., Daly M., Walsh W.P., Resh N., Seay J., Barsevick A., Birenbaum L., Heggan T., Martin G. (1993b), Communication between patients with breast cancer and health care providers: Determinants and implications, *Cancer*, 72: 2612-2620.

Lerman C., Kash K., Stefanek M. (1994b), Younger women at increased risk for breast cancer: Perceived risk, psychological well-being, and surveillance, *Journal of the National Cancer Institute Monographs*, 16: 171-176.

Lerman C., Miller S.M., Scarborough R., Hanjani P., Nolte S., Smith D. (1991a), Adverse psychologic consequences of positive cytologic cervical screening, *American Journal of Obstetrics and Gynecology*, 165: 658-662.

Lerman C., Rimer B., Blumberg B., Cristinzio S., Engstrom P.E. MacElwee N., O'Conner K., Seay J. (1990), Effects of coping style and relaxation on cancer chemotherapy side-effects and emotional responses, *Cancer Nursing*, 13: 308-315.

Lerman C., Trock B., Rimer B., Boyce A., Jepson C., Engstrom P.E. (1991b), Psychological and behavioral implications of abnormal mammograms, *Annals of Internal Medicine*, 114: 657-661.

Leventhal H., Diefenbach M., Leventhal E. (1992), Illness cognition: Using common sense to understand treatment adherence and affect cognition interaction, *Cognitive Therapy and Research*, 16: 143-163.

Litt M. Nye C., Shafer D. (1995), Preparation for oral surgery: Evaluating elements of coping, *Journal of Behavioral Medicine*, 18: 435-459.

Meichenbaum D. (1992), *Stress inoculation training: A twenty-year update,* in Woolfolk R.L., Lehrer P.M. (eds.), *Principles and practice of stress management*, Guilford Press, New York.

Miller S.M. (1987), Monitoring and *blunting*: Validation of a questionnaire to assess styles of information seeking under threat, *Journal of Personality and Social Psychology*, 52: 345-353.

Miller S.M. (1995), Monitoring versus *blunting* styles of coping with cancer influence the information patients want and need about their disease (implications for cancer screening and management), *Cancer*, 76: 167-177.

Miller S.M. (1996a), *Monitoring and blunting of threatening information: Cognitive interference and facilitation in the coping process,* in Sarason I.G., Pierce G.R., Sarason B.R. (eds.), *Cognitive interference: Theories, methods, and findings*: 175-190, Lawrence Erlbaum, NJ.

Miller S.M., Brody D., Summerton J. (1988), Styles of coping with threat: Implications for health, *Journal of Personality and Social Psychology*, 54: 223-236.

Miller S.M., Buzaglo J.S., Simms S., Green V.A., Bales C.B., Mangan C.E., Sedlacek T.V. (1999a), Monitoring styles in women at risk for cervical cancer: Implications for the framing of health-relevant messages, *Annals of Behavioral Medicine*, 21: 91-99.

Miller S.M., Diefenbach M.A. (1998a), *The cognitive-social health information processing (C-SHIP) model: a theoretical framework for research in behavioral oncology*, in Krantz D. (ed.), *Perspectives in behavioral medicine*: 219-244, Erlbaum, Hillsdale, NJ.

Miller S.M., Fang C.Y., Diefenbach M.A., Bales C.B. (in press), *Tailoring psychosocial interventions to the individual's health information-processing style: The influence of monitoring versus blunting in cancer risk and disease*, in Baum A., Anderson B. (eds.), *Psychosocial interventions and cancer*, American Psychological Association, Washington, D.C.

Miller S.M., Fang C.Y., Manne S.L., Engstrom P.E., Daly M.B. (1999b), Decision making about prophylactic oophorectomy among at-risk women: Psychological influences and implications, *Gynecologic Oncology*, 75: 406-412.

Miller S.M., Green V.A., Bales C.B. (1999c), *What you don't know can hurt you: A cognitive-social framework for understanding children's responses to stress*, in Lewis M. and Ramsay D. (eds.), *Soothing and Stress: 257-292*, Lawrence Erlbaum Mahwah, NJ.

Miller S.M., Mangan C.E. (1983), The interacting effects of information and coping style in adapting to gynecologic stress: Should the doctor tell all? *Journal of Personality and Social Psychology*, 45: 223-236.

Miller S.M., Mischel W., O'Leary A., Mills M. (1996b), From human papillomavirus (HPV) to cervical cancer: Psychological processes in infection, detection, and control, *Annals of Behavioral Medicine*, 18: 219-228.

Miller S.M., Mischel W., Schroeder C.M., Buzaglo J., Hurley K., Schreiber P., Mangan C.E., Sedlacek T.V. (1998b), Intrusive and avoidant ideation among females pursuing infertility treatment, *Psychology and Health*, 13: 847-858.

Miller S.M., Rodoletz M., Buzaglo J.S., Sherman K.A., Gray T. (2001), Monitoring style in low-income minority women: Cognitive social determinants of adjustment and adherence, *Society for Behavioral Medicine*, Seattle, Washington.

Miller S.M., Rodoletz M., Schroeder C.M., Mangan C.E., Sedlacek T.V. (1996c), Applications of the *monitoring* process model to coping with severe long-term medical threats, *Health Psychology*, 15: 216-225.

Miller S.M., Roussi P., Altman D., Helm W., Steinberg A. (1994), The effects of coping style on psychological reactions to colposcopy among low-income minority women, *Journal of Reproductive Medicine*, 39: 711-718.

Miller S.M., Seijak K.K., Schroeder C.M., Lerman C., Hernandez E., Helm C.W. (1997), Enhancing adherence following abnormal Pap smears among low-income minority women: A preventive telephone counseling strategy, *Journal of the National Cancer Institute*, 89: 703-708.

Miller S.M., Schnoll R.A. (2000), *When seeing is feeling: A cognitive-emotional approach to*

coping with health stress, in Lewis M., Haviland-Jones J.M. (eds.), *Handbook of emotions, 2nd edition:* 538-557.
Miller S.M., Shoda Y., Hurley K. (1996d), Applying cognitive-social theory to health-protective behavior: Breast self-examination in cancer screening, *Psychological Bulletin*, 119: 70-94.
Muris P., van Zuuren F.J. (1992), Monitoring, medical fears and physical symptoms, *British Journal of Clinical Psychology*, 31: 360-362.
Phipps S., Srivastava D.K. (1997), Repressive adaptation in children with cancer, *Health Psychology*, 16: 521-528.
Rees C.E., Bath P.A. (2000), The psychometric properties of the Miller Behavioral Style Scale with adult daughters of women with early breast cancer: A literature review and empirical study, *Journal of Advanced Nursing*, 32: 366-374.
Rothman A.J., Salovey P. (1997), Shaping perceptions to motivate healthy behavior: the role of message framing, *Psychological Bulletin*, 121: 3-19.
Sarason I. (1979), Three lacunae of cognitive therapy, *Cognitive Therapy and Research*, 3: 223-235.
Scheier M., Carver C. (1985), Optimism, coping, and health: Assessment and implications of generalized outcome expectancies, *Health Psychology*, 4: 219-247.
Schwartz M.D., Lerman C., Miller S.M., Daly M., Masny A. (1995), Coping disposition, perceived risk, and psychological distress among women at increased risk for ovarian cancer, *Health Psychology*, 14: 232-235.
Sherman K.A. (1999), The role of information in women's breast screening, *Abstracts of the 34th Annual Conference of the Australian Psychological Society, Australian Journal of Psychology*, 51(Supplement): 138.
Stefanek M.E., Wilcox P. (1991), First degree relatives of breast cancer patients: screening practices and provision of risk information, *Cancer Detection Prevention*, 15: 379-384.
Steptoe A., O'Sullivan J. (1986), Monitoring and *blunting* coping styles in women prior to surgery, *British Journal of Clinical Psychology*, 25: 143-144.
Steptoe A., Sutcliffe I., Allen B., Coombes C. (1991), Satisfaction with communication, medical knowledge, and coping style in patients with metastatic cancer, *Social Science and Medicine*, 32: 627-632.
Taylor S.E. (1990), Health psychology: The science and the field, *American Psychologist*, 45: 40-50.
Wardle J. (1995): Women at risk of ovarian cancer, *J. of the National Cancer Institute Monographs*, 17: 81-85.

Traduzione curata dalla dott.ssa Carlotta Scaramuzzi

PSYCHOSOCIAL DETERMINANTS OF PARTICIPATION IN RISK COUNSELING PROGRAMS AND BREAST CANCER SCREENING REGIMENS AMONG AFRICAN AMERICAN WOMEN

(Heading: Psychosocial determinants of participation)

Kerry A. Sherman, Ph.D.
Assistant Member
Division of Population Science
Fox Chase Cancer Center
Philadelphia, PA

Suzanne M. Miller, Ph.D.
Senior Member, Division of Population Science
Director, Behavioral Medicine Program
Director, Behavioral Research Core Facility
Fox Chase Cancer Center
Philadelphia, PA

Sherri Sheinfeld Gorin, Ph.D. [a]
Assistant Professor of Sociomedical Sciences and Epidemiology
Mailman School of Public Health of Columbia University
600 West 168th Street, fourth floor
New York, N.Y. 10032
212-305-1185
Fax: 212-305-9413
E-mail: ssg19@columbia.edu

[a] Address for reprints

This research was supported in part by Grant BCTR-2000-657 from the Komen Foundation (S. Sheinfeld Gorin, PI), and Grants DAMD 17-01-1-0238, DAMD 17-02-1-0382, and DAMD 17-98-1-8306 from the Department of Defense (M. Daly, S. Miller, PI's)

ABSTRACT

Purpose. Since most of the research about the uptake and impact of risk assessment programs has focused on Caucasian women, little data are available to guide the implementation of these programs specifically addressing the unique needs of African American women. Further, no published reviews have yet integrated the cognitive-affective and social support constructs that predict participation in breast cancer screening among African American women. In this paper we outline two unifying theoretical framework, the Cognitive-Social Health Information Processing (C-SHIP), for understanding genetic risk assessment participation and breast screening behaviors among African American women.

Description of the Study. The influence of each of the four distinctive cognitive-emotional processes that underlie the information processing of cancer risk information on the uptake of genetic testing and screening participation among African American women is explored. These include: 1) individuals' self-construals of their risk, including their knowledge levels and perceived risk; 2) their expectancies about the benefits and limitations of specific cancer-related actions; 3) their health values (e.g., fatalistic attitudes about cancer); and, 4) cancer-specific emotional distress. Using the Social Network approach, the effect of social support, involving actual or perceived exchanges of emotional (e.g., esteem, trust), appraisal (e.g., affirmation, social comparison), informational (e.g., advice, directives) and instrumental support, are examined as influences on participation by black women in risk assessment programs and surveillance for breast cancer.

Results. The accuracy of risk perceptions among average risk African American woman is not related to breast cancer screening adherence. However, among at-risk women, accurate knowledge of breast cancer risk is associated with screening practices, albeit at less than recommended frequencies.

Beliefs that early detection can lead to cure of breast cancer, and belief in taking charge of one's own health are associated with increased mammography utilization, while perceived barriers related to mammography (e.g., fear of radiation, perceptions of lack of healthcare access) are associated with decreased mammography use.

Within the genetic risk assessment context, fatalistic thinking among African Americans concerning the inevitability of cancer diagnosis is related to low levels of interest and low likelihood of participation in such programs.

Under routine stress conditions, high monitors (who are more attentive to health threats) demonstrate increased adherence to recommended screening regimens. Under conditions of high threat (e.g., feedback of an abnormal screening result), however, high monitors respond with heightened levels of risk-related anxiety and avoidance.

Considerable evidence supports the importance of the social support system (i.e., family, friends, physicians) in enhancing risk assessment decisions and in increasing breast screening adherence among African American women.

Implications. Counseling strategies may be more effective if they are systematically tailored to the specific cognitive and affective profiles of African American women as they decide about participating in risk assessment programs and adhering to breast cancer screening regimens. Concomitantly, the social support networks within which African American women live could be mobilized to encourage the understanding of risk and to develop community norms that encourage longer-term adherence and more informed decisions.

BREAST CANCER INCIDENCE, MORTALITY AND SURVEILLANCE AMONG AFRICAN-AMERICANS

It is estimated that 203,500 women will be diagnosed with breast cancer in 2002 and 40,000 women will die from the disease (1). Although Caucasians are more likely to develop breast cancer (i.e., 115.5 cases for every 100,000 Caucasian women, compared with 101.5 African American cases), African American women are more likely to die from the disease (2,3). It is well documented that inadequate cancer screening leads to later stage disease (4).

Higher mortality rates among African American women may be due, in part, to the fact that they are less likely to adhere to breast screening recommendations (4, 5), particularly among older women (6, 7), and that they more often delay seeking treatment in the presence of symptoms (8). Incidence of poor mammography adherence is particularly prevalent among low income and underinsured women (9). While breast cancer screening rates among African American women have risen in recent years, they are still below optimal levels (10). Thus, one factor accounting for differential morbidity and mortality rates among African American and Caucasian women may be variations in breast cancer screening adherence, with African Americans more likely to be among the lower income, less educated, and inadequately insured group (11, 12).

In addition to the availability of screening regimens, the recent discovery and identification of the *BRCA1* and *2* genes associated with inherited susceptibility to breast and ovarian cancer has led to the implementation of breast cancer familial risk assessment programs. *BRCA1/2* mutations account for approximately 5% of breast cancer cases (1), and confer an estimated range from 36-85% lifetime risk of developing breast cancer, and a 16-60% lifetime risk for ovarian cancer (13). Recent investigations of *BRCA1/2* mutations in African American

populations have identified similar rates of these mutations as have been previously identified in Caucasian populations (14, 15). Familial risk assessment programs generally entail cancer risk education, personal pedigree feedback, genetic risk assessment for putative hereditary cases, and individualized screening recommendations.

African American women are notably under-represented in familial risk programs, compared with Caucasian women (12,16). Even when African American women participate in these programs, they appear to derive less benefit from their participation compared with Caucasian women. For example, one study (16) found that African American women did not exhibit as large an increase in their levels of pros about risk assessment nor as substantial a decrease in their levels of cons about assessment, versus white women after risk assessment counseling. One explanation for these results is that assessment programs have been created in a generic fashion, failing to consider unique barriers and facilitators to assessment for African American women (12). Since most of the research about the uptake and impact of risk assessment programs has focused on Caucasian women, little data are available to guide the implementation of risk assessment programs specifically addressing the unique needs of African American women. Similarly, although several comprehensive reviews have isolated the major predicators of breast cancer screening among African American women (17,18,19,20,21), the findings have not been incorporated within a unifying theoretical framework.

In this paper we outline The Cognitive-Social Health Information Processing (C-SHIP) model for understanding genetic risk assessment participation and breast screening behaviors among African American women. We review the cognitive-affective factors that account for variability in women's responses to cancer risk, both in terms of adherence to cancer screening and in interest in genetic risk assessment participation. We then provide two integrative

perspectives on this literature by focusing on signature attentional styles of processing health-related information (i.e., high versus low monitoring) and the social network model. Finally, we discuss implications of these findings and future directions to address breast screening adherence and genetic risk assessment participation among African American women.

A COGNITIVE-SOCIAL THEORY FOR BREAST CANCER SURVEILLANCE BEHAVIOR

The Cognitive-Social Health Information-Processing model (C-SHIP; 22) is a broad theoretical framework that informs the design and assessment of behavior change treatments in the context of cancer prevention and control (23). The model postulates that individuals can be characterized by their cognitive and affective responses to health-relevant threats, and it is these responses that determine their dispositional tendencies or "behavioral signatures" towards health-enhancing vs. health-diminishing behaviors (24, 25). The C-SHIP model has been widely used to conceptualize behavior change in diverse health contexts, including cancer risk (22, 25, 26).

According to the model, there are four distinctive cognitive-emotional processes that underlie the information processing of cancer risk information: 1) individuals' self-construals of their risk, including their knowledge levels and perceived risk; 2) their expectancies about the benefits and limitations of specific cancer-related actions; 3) their health values (e.g., fatalistic attitudes about cancer); and, 4) cancer-specific emotional distress. Each of the four C-SHIP cognitive-emotional processes will now be systematically reviewed with respect to breast screening and genetic risk assessment related behaviors among African American women.

Breast Cancer Related Self-Construals: Knowledge and Perceived Risk

Women who lack sufficient knowledge of available surveillance regimens cannot take advantage of them (e.g., 27). Mammography adherence is generally related to knowledge about breast cancer risk and recommendations, such that higher levels of knowledge predict greater utilization (28, 29, 30, 31). Among African American women, levels of breast cancer related knowledge are generally low. Therefore, it is not surprising that adherence to screening recommendations is less than optimal for these women (5,32).

Perceived risk for breast cancer is another factor that influences participation in risk assessment and screening programs among African American women. Studies examining perceived risk for breast cancer among African American women at average risk for breast cancer have yielded inconsistent results. Although there is some evidence for underestimation of risk (33, 34), on balance, there is more evidence to suggest that African American women overestimate their risk for breast cancer relative to Caucasian and Hispanic women (12; 35, 36). Further, among at-risk African American women with a family history of breast cancer, perceived breast cancer risk is greater than for average risk African American women (37), but lower than for at-risk Caucasian women (38, 67). Increased perceptions of risk have been linked with increased worries about the affected relative, and younger age among women at increased risk for breast cancer (38,35,39). Inaccurate risk estimates among African American women may be attributed to lower levels of cancer breast cancer-related knowledge (40), and risk-related intrusive thoughts interfering with the processing of risk information, (41).

The accuracy of risk perceptions among average risk African American woman is not related to breast cancer screening adherence (71). However, among at-risk women, accurate knowledge of breast cancer risk is associated with screening practices, albeit at less than

recommended frequencies (8). Further, African Americans who overestimate their risk may even engage in excessive breast self examination (38); a less effective screening approach than mammography.

Lower perceived risk is related to decreased interest in genetic testing among African American women (42,12,37). Accuracy of risk assessment may be improved through educational intervention (41, 43), and, when risk information is provided within the context of a trusting relationship, interest in genetic testing may increase (44). However, actual uptake of genetic testing does not appear to necessarily follow expressions of interest (44).

Breast Cancer Related Expectancies and Beliefs

Perceived benefits (i.e., pros) and limitations (i.e., cons) about breast cancer risk have been associated with health-protective behaviors (45). In the screening context, a number of studies conducted among predominantly Caucasian women have documented a relationship between women's perceived benefits of mammography and their utilization adherence (e.g., 29, 46, 28, 47, 30, 31, 48). Conversely, perceived barriers (e.g., expectations of pain) have been consistently associated with reduced mammography adherence (e.g., 29, 46, 31, 49). Similarly, in studies specifically focusing on African American women, beliefs that early detection can lead to cure of breast cancer, and belief in taking charge of one's own health (50) are associated with increased mammography utilization (51, 32), while perceived barriers related to mammography (e.g., fear of radiation, perceptions of lack of healthcare access) are associated with decreased mammography use (52, 53, 54, 55). Utilization of clinical breast examination within African American subgroups (US-born African Americans and English-speaking Afro-Caribbeans) is associated with belief in the efficacy of clinical exams, whereas infrequent use is associated with lack of trust in the efficacy of cancer treatments (Carol Magai, personal communication).

The little research conducted with African American women in the genetic risk assessment context suggests that readiness to participate in genetic risk assessment can be largely attributed to perceptions that the advantages of testing outweigh the disadvantages (56). Indeed, a recent study shows that African American women are interested in genetic risk assessment to the extent that they believe that testing outcomes would be of benefit to themselves and to their families (57). Further, despite having lower levels of knowledge, African American women report more positive attitudes about the benefits of genetic risk assessment (42,58). On the other hand, African American women perceive the cost and availability of *BRCA1/2* testing as salient barriers to participation (42, 57).

Breast Cancer Related Values and Goals

Fatalistic beliefs (i.e., believing, for instance, that there is no use in getting tested or screened, since cancer is inevitable) are prevalent among African American women, particularly those who are lower income, less well-educated, and unemployed (59, 60). Individuals who are characterized by higher levels of fatalistic beliefs report significantly lower levels of adherence to screening for breast cancer, particularly among low-income (61) and Afro-Caribbean women (62, 63, Carol Magai, personal communication). Fatalistic attitudes, like other shared national values and belief systems (64, 65, 59), act by lowering self-efficacy and expectancies about the outcomes of health-related behaviors, in turn reducing the motivation for an individual to persist in enacting specific health-protective behaviors such as mammography (22). Within the genetic risk assessment context, fatalistic thinking among African Americans concerning the inevitability of cancer diagnosis is related to low levels of interest and low likelihood of participation in such programs (57). In a related vein, women with high levels of spiritual faith, that is women who believe that a higher power determines who gets cancer, (who are, therefore,

presumably less reliant on the medical system) report less likelihood of participating in genetic risk assessment (66).

Breast Cancer Related Emotional Distress

Among women at increased risk for breast cancer with at least one first degree relative (FDR) diagnosed with breast cancer, African American women report significantly greater concerns and worries about their affected relative, and heightened avoidance of breast-cancer related thoughts and feelings compared with Caucasian women (67). With respect to screening behaviors, some research points to a positive relationship between breast cancer related distress and adherence (e.g., 68, 69), whereas other research points to a negative relationship (e.g., 70, 71). In studies linking higher distress with greater screening adherence, cancer worry was at a moderate level, whereas cancer worry reported in Caryn Lerman and colleagues' (70, 71) studies was at a level that interfered with daily functioning. Therefore, an inverted U-shaped relationship between distress and screening behavior appears to exist, whereby moderate levels of cancer worry motivate screening behavior, but extremely high or low levels of psychological distress inhibit cancer screening (22, 23). Within the African American community, fear of cancer is reported as one of the most important reasons for not participating in mammography screening programs (72, 73). However, among FDRs of African American women with breast cancer, fear of cancer is also associated with excessive use of breast self-examination (74).

Interest in genetic risk assessment is associated with increased levels of cancer-related emotional distress (75, 76), but decreased understanding and comprehension of genetic risk feedback (e.g., 12). Moreover, fears about how to emotionally deal with genetic risk feedback are central reasons reported by African Americans for not wanting to participate in genetic risk assessment (57).

ATTENTIONAL STYLE

According to the C-SHIP model, individuals are characterized by distinctive styles or behavioral signatures in how they select, encode, and manage health information, and how they react to it (24). In particular, these behavioral signature attentional response styles have been systematically explored in terms of *high monitors* (who scan for, and magnify, threatening cues) and *low monitors* (who distract from, and downgrade, threatening information; 24). There is evidence to support the universality of these behavioral signatures, with consistent effects emerging across diverse ethnic (e.g., Caucasians vs. African Americans) and cultural groups (e.g., European versus American) (24, 77). The two attentional styles are characterized by different organizations in the structure of their self-construals, beliefs, and behaviors (77).

Under routine stress conditions, high monitors (who are more attentive to health threats) are more likely to behave in an adaptive fashion, demonstrating increased adherence to recommended screening regimens (78). However, under conditions of high threat (e.g., feedback of an abnormal screening result) high monitors respond with heightened levels of risk-related anxiety and avoidance (79, 66). This response can ultimately undermine and interfere with their ability to maintain required health-protective behaviors (22, 80). In contrast, low monitors -- who are less focused on health threats -- are likely to ignore cancer threats from the outset and thereby demonstrate poor screening adherence overall (22).

Attentional style also appears to be an important determinant of interest in genetic risk assessment and testing. High monitors are more likely to report increased perceived risk for breast cancer, more pessimistic attitudes about their vulnerability to breast cancer, and increased breast cancer-related distress, compared with low monitors (24, 66, 81, 82). Hence, they are more likely to express greater interest in participating in genetic risk assessment and testing.

However, expectations of increased adverse psychological consequences following testing can be salient barriers that influence the genetic risk assessment decision-making processes for high monitors (75). Similarly, patterns of communication of genetic risk assessment results to family members are influenced by attentional style. Among women with a putative hereditary breast cancer pattern undergoing breast/ovarian genetic risk assessment, high monitors reported greater intentions to communicate positive test results to family members and to engage in strategic planning for this communication (83). Taken together, these results suggest that interventions designed to promote breast screening and appropriate uptake of genetic risk assessment programs need to take account of the unique cognitive-affective profile of the individual (26, 84), as well as the unique characteristics of the African American culture.

SOCIAL SUPPORT

Considerable evidence supports the importance of the social support system (i.e., family, friends, physicians) in increasing breast screening adherence among African American women; referred to here as the *Social Network* perspective (see 85, 86, 87). African American women are embedded within a context of nested systems, families, peer groups, organizations (e.g., medical centers, worksites) and larger communities (88). Within this context, the woman creates a network of unique relationships with which she exchanges emotional (e.g., esteem, trust), appraisal (e.g., affirmation, social comparison), informational (e.g., advice, directives) and instrumental support (e.g., money, time) (89, 90). These support sources form the most salient norms and values to which the woman responds, as well as critical information convoys, subsequently influencing her breast cancer surveillance behaviors. As a source of accurate information, the physician may also influence screening and risk assessment decisions and behaviors (91,92,93,94). Physicians, however, are less likely to encourage breast cancer

screening (95, 96) or to discuss genetic testing (42) with African Americans than with other women.

African American women are also particularly likely to experience the barrier of having social networks who are fearful of orthodox medical care, and thus not able to encourage the women to engage in breast cancer surveillance behaviors (97). For example, the screening practices of relatives and friends has been found to influence surveillance among African American women (98,99). Further, participation in breast screening has been linked with increased number of social ties (100), and being married or living with a partner (101). Although the data are limited, the participation of African American FDR's in risk assessment programs seems related to their concerns for, and care of their affected relative (67).

IMPLICATIONS AND FUTURE DIRECTIONS

It is increasingly recognized that psychosocial interventions can be effective in promoting appropriate interest in, and use of, cancer risk assessment programs, as well as promoting screening adherence (84, 102). However, the specific content and structure of these interventions are just beginning to be linked to state-of-the-art theory and research (103,16). Risk counseling protocols have been shown to reduce breast cancer specific distress, knowledge of risk, and risk perception, particularly for FDRs with less education (79), thereby improving program attendance as well as increasing adherence to screening recommendations (103, 104, 105). However, some data suggest that generic interventions, developed for Caucasian women, are not effective with underserved populations (e.g., 12, 16).

The C-SHIP model offers a framework for understanding how African American women differentially seek, amplify, and process different types of risk information, provided by multiple sources (22). Counseling strategies may be more effective if they are systematically tailored to

the specific cognitive and affective profiles of African American women as they deal with the challenge of engaging in breast cancer screening and making decisions about participation in risk assessment programs. Concomitantly, the social support networks within which African American women live could be mobilized to encourage the understanding of risk and to develop community norms that encourage more informed decisions and longer-term adherence.

REFERENCES

1 American Cancer Society. (2002). Cancer Facts and Figures 2002. Atlanta:ACS.

2 Landis, S., Murray, T., Bolden, S., & Wingo, P.A. (1999). Cancer Statistics, 1999. CA A Cancer Journal for Clinicians, 49, 8-31.

3 Russell, A., Langlois, T., Johnson, G., Trentham-Dietz, A., & Remington, P. (1999). Increasing gap in breast cancer mortality between black and white women. WMJ, 98, 37-39.

4 Burack, R. C. Liang, J. (1989). The acceptance and completion of mammography in older black women. American Journal of Public Health, 79, 721-726.

5 Chu, K.C., Tarone, R.E., & Brawley, O.W. (1999). Breast cancer trends of black women compared with white women. Archives of Family Medicine, 8, 521-528.

6 Burns, R.B., McCarthy, E.P., Freund, K.M., Marwill, S.L., Shwartz, M., Ash, A., Moskowitz, M.A. (1996). Variability in mammography use among older women. Journal of the Amreican Geriatrics Society, 44, 922-6.

7 Mandelblatt, J., Traxler, M., Lakin, P., Kanetsky, P., & Kao, R. (1992). Mammography and Papanicolaou smear use by elderly poor black women. The Harlem Study Team. Journal of the Amreican Geriatrics Society, 40, 1001-7.

8 Royak-Schaler, R., deVellis, B.M., Sorenson, J.R., Wilson, K.R., Lannin, D.R., & Emerson, J.A. (1995). Breast cancer in African-American families. Risk perception, cancer worry, and screening practices of first-degree relatives. Annals of the New York Academy of Science, 768, 281-285.

9 Blackman DK, Bennett EM, Miller DS. (1999). Trends in self-reported use of mammograms (1989-1997) and Papanicolaou tests (1991-1997)—Behavioral Risk Factor Surveillance System. MMWR Weekly, 48, 1-22.

10 Crump, S.R., Mayberry, R.M., Taylor, B.D., Barefield, K.P., & Thomas, P.E. (2000). Factors relating to noncompliance with screening mammogram appointments among low-income African American women. Journal of the National Medical Association, 92, 237-246.

11 Baker, F. (1999). Some reflections on racial profiling. Journal of the American Academy of Psychiatry Law, 27, 626-629.

12 Bowen, D., Hickman, K., & Powers, D. (1997). Importance of psychological variables in understanding risk perceptions and breast cancer screening of African American women. Women's Health Research in Gender Behavior and Policy, 3, 227-242.

13 Daly, M. (1999). NCCN practice guidelines: genetics/familial high-risk cancer screening. Oncology, NCCN Proceedings, 161-183.

14 Gao, Q., Tomlinson, G., Das, S., Cummings, S., Sveen, L., Fackenthal, J., & Schumm, L. (2000). Prevalence of BRCA1 and BRCA2 mutations among clinic-based African American families with breast cancer. Human Genetics, 107, 186-191.

15 Shen, D., Wu, Y., Subbarao, M., Bhat, H., Chillar, R., & Vadgama, J.V. (2000). Journal of the National Medical Association, 92, 29-35.

16 Lerman, C., Biesecker, B, Benkendorf, JL, Kerner, J, Gomez-Caminero, A, Hughes, C, Reed, MM. (1997). Controlled trial of pretest education approaches to enhance informed decision-making for BRCA1 gene testing. Journal of the National Cancer Institute, 89, 148-57.

17 Hoffman-Goetz, L., Mills, S.L. (1997). Cultural barriers to cancer screening among African American women: a critical review of the qualitative literature. Women's Health, 3, 183-201.

18 Ashing-Giwa, K. (1999). Health behavior change models and their socio-cultural relevance for breast cancer screening in African American women. Women Health, 28, 53-71.

19 Facione, N.C., & Katapodi, M. (2000). Culture as an influence on breast cancer screening and early detection. Seminars in Oncology Nursing, 16, 238-47.

20 Yabroff, K.R., & Mandelblatt, J.S. (1999). Interventions targeted toward patients to increase mammography use. Cancer Epidemiology, Biomarkers, and Prevention, 8, 749-57.

21 Legler, J., Meissner, H.I., Coyne, C., Breen, N., Chollette, V., & Rimer, B.K. (2002). The effectiveness of interventions to promote mammography among women with historically lower rates of screening. Cancer Epidemiology, Biomarkers, and Prevention, 11, 59-71.

22 Miller, S. M., Shoda, Y., & Hurley, K. (1996). Applying cognitive-social theory to health-protective behavior: Breast self-examination in cancer screening. Psychological Bulletin, 119, 70-94.

23 Miller, S. M., Mischel, W., O'Leary, A., Mills, M. (1996). From human papillomavirus (HPV) to cervical cancer: psychosocial processes in infection, detection, and control. Annals of Behavioral Medicine, 18, 219-228.

24 Miller, S.M. (1995). Monitoring versus blunting styles of coping with cancer influence the information patients want and need about their disease (implications for cancer screening and management). Cancer, 76, 167-177.

25 Shoda, Y., Mischel, W., Miller, S. M., Diefenbach, M., Daly, M. B., Engstrom, P. F. (1998). Psychological interventions and genetic testing: Facilitating informed decisions about BRCA1/2 cancer susceptibility. Journal of Clinical Psychology in Medical Setting, 5, 3-17.

26 Miller, S.M., Fang, C.Y., Manne, S.L., Engstrom, P.E., & Daly, M.B. (1999). Decision making about prophylactic oophorectomy among at-risk women: Psychological influences and implications. Gynecologic Oncology, 75, 406-412.

27 Womeodu, R., & Bailey, J. (1996). Barriers to cancer screening. Medical Clinics of North America, 80, 115-33.

28 Costanza, M. E. (1994). The extent of breast cancer screening in older women. Cancer, 74, 2046-2050.

29 Champion, V., & Miller, A. M. (1996). Recent mammography in women aged 35 and older: predisposing variables. Health Care Women International, 17, 233-245.

30 Miller, A. M., & Champion, V. L. (1996). Mammography in older women: one-time and three-year adherence to guidelines. Nursing Research, 45, 239-245.

31 Savage, S. & Clarke, V.A. (1996). Factors associated with screening mammography and breast self-examination intentions. Health Education Research, 11, 409-421.

32 Skinner, C.S., Arfken, C.L., & Sykes, R.K. (1998). Knowledge, perceptions, and mammography stage of adoption among older urban women. American Journal of Preventive Medicine, 14, 54-63.

33 Glanz, K, Resch, N., Lerman, C., Rimer, B.K. (1996). Black-white differences in factors influencing mammography use among employed female health maintenance organization members. Ethnicity and Disease, 1, 207-20.

34 Barroso, J., McMillan, S., Casey, L., Gibson, W., Kaminski, G., Meyer, J. (2000). Comparison between African-American and white women in their beliefs about breast cancer and their health locus of control. Cancer Nursing 23, 268-76.

35 Vernon, S.W., Victor, V.G., Halabi, S., & Bondy, M. (1993).Factors associated with perceived risk of breast cancer among women attending a screening program. Breast Cancer Research and Treatment, 28, 137-44.

36 Helzlsouer, K.J., Ford, D.E., Hayward, R.S.A., Midzenski, M., & Perry, H. (1994). Perceived risk of cancer and practice of cancer prevention behaviors among employees in an oncology center. Preventive Medicine, 23, 302-308.

37 Lipkus, I. Lipkus, I. M., Iden, D., Terrenoire, J., & Feaganes, J. R. (1999). Relationships among breast cancer concern, risk perceptions, and interest in genetic testing for breast cancer

susceptibility among African American women with and without a family history of breast cancer. Cancer Epidemiology, Biomarkers, and Prevention, 8, 533-539.

38 Audrain, J., Lerman, C., Rimer, B., Cella, D., Steffens, R., Gomez-Caminero, A., & the High Risk Breast Cancer Consortium. (1995). Awareness of heightened breast cancer risk among first-degree relatives of recently diagnosed breast cancer patients. Cancer Epidemiology Biomarkers and Control, 4, 561-65.

39 Daly, M.B., Lerman, C.L., Ross, E., Schwartz, M., Sands, C.B., & Masny, A. (1996). Gail model breast cancer risk components are poor predictors of risk perception and screening behavior. Breast Cancer Research and Treatment, 41, 59-70.

40 Rapp, R. (1999). Amniocentesis in sociocultural perspective. Journal of Genetic Counseling, 2, 183-96.

41 Lerman, C., Lustbader, E., Rimer, B., Daly, M., Miller, S., Sands, C., Balshem, A. (1995). Effects of individualized breast cancer risk counseling: a randomized trial. Journal of the National Cancer Institute, 87, 286-92.

42 Kinney, A.Y., Croyle, R.T., Dudley, W.N., Bailey, C.A., Pelias, M.K., & Neuhausen, S.L. (2001). Knowledge, attitudes, and interest in breast-ovarian cancer gene testing: a survey of a large African-American kindred with a BRCA1 mutation. Preventive Medicine, 33, 543-551.

43 Lipkus, I., Klein, W.P., & Rimer, B.K. (2001). Communicating breast cancer risks to women using different formats. Cancer Epidemiology, Biomarkers, and Control, 10, 895-8.

44 Lerman, C., Hughes, C., Beckendorf, J.L., Biesecker, B., Kerner, J., Willson, J., Eads, N., Hadley, D., & Lynch, J. (1999). Racial differences in testing motivation and psychological distress following pretest education for BRCA1 gene testing. Cancer Epidemiology, Biomarkers, and Prevention, 8, 361-67.

45 Lerman, C., Narod, S., Schulman, K., Hughes, C., Gomez-Caminero, A., Bonney, G., Gold, K., Trock, B., Main, D., Lynch, J., Fulmore, C., Snyder, C., Lemon, S., Conway, T., Tonin, P., Lenior, G., & Lynch, H. (1996). BRCA1 testing in families with hereditary breast-ovarian cancer: A prospective study of patient decision making and outcomes. Journal of the American Medical Association, 275, 1885-1892.

46 Champion, V. L. Scott, C.R. (1997). Reliability and validity of breast cancer screening belief scales in African American women. Nursing Research, 46, 331-337.

47 Holm, C. J., Frank, D. I., & Curtin, J. (1999). Health beliefs, health locus of control, and women's mammography behavior. Cancer Nursing, 22, 149-156.

48 Thomas, L. R., Fox, S. A., Leake, B. G., & Roetzheim, R. G. (1996). The effects of health beliefs on screening mammography utilization among a diverse sample of older women. Women Health, 24, 77-94.

49 Caruso, A., Efficace, F., Parrila, A., Angelone, L., Ferranti, F., & Grandinetti, M.L. (2001). Pain and anxiety related to mammography in breast cancer patients. Psychological evaluation in an experiemental study. Radiological Medicine, 102, 335-339.

50 Milburn, N., Magai, C., Cohen, C., & Fyffe, D. (August, 1998). Intra-group differences in health beliefs and attitudes among older Black women. In L.Beatty (Chair), Developing effective health interventions for Black women. Symposium presented at the American Psychological Association, San Francisco.

51 Husaini, B.A., Sherkat, D.E., Bragg, R., Levine, R., Emerson, J.S., Mentes, C.M., & Cain, V.A. (2001). Predictors of breast cancer screening in a panel study of African American women. Women and Health, 34, 35-51.

52 Glanz, K., Resch, N., Lerman, C., & Rimer, B.K. (1996). Black-white differences influencing mammography use among employed female health maintenance organization members. Ethnicity and Health, 1, 207-220.

53 Facione, N.C. (1999). Breast cancer screening in relation to access to health services. Oncologic Nursing Forum, 26, 689-696.

54 Brown, L.W., & Williams, R.D. (1994). Culturally sensitive breast cancer screening programs for older black women. Nurse Practitioner, 19, 21-26.

55 Phillips, J.M., & Wilbur, J. (1995). Adherence to breast cancer screening guidelines among African-American women of differing employment status. Cancer Nursing, 18, 258-269.

56 Jacobsen, P. B., Valdimarsdottier, H. B., Brown, K. L., & Offit, K. (1997). Decision-making about genetic testing among women at familial risk for breast cancer. Psychosomatic Medicine, 59, 459-466.

57 Matthews, A.K., Cummings, S., Thompson, S., Wohl, V., List, M., & Olopade, O. (2000). Genetic testing of African Americans for susceptibility to inherited cancers: use of focus groups to determine factors contributing to participation. Journal of Psychosocial Oncology, 18, 1-19.

58 Hughes, C., Gomez-Caminero, A, Benkendorf, J, Kerner, J, Isaacs, Barter, J & Lerman, C. (1997). Ethnic differences in knowledge and attitudes about BRCA1 testing in women at increased risk. Patient Education and Counseling, 32, 51-62.

59 Phillips, J. M., Cohen, M.Z., & Moses, G. (1999). Breast cancer screening and African American Women: Fear, Fatalism, and Silence. Oncology Nursing Forum, 26, 561-571.

60 Powe, B. D., & Weinrich, S. (1999). An intervention to decrease cancer fatalism among rural elders. Oncology Nursing Forum, 26, 583-8.

61 Suarez, L., Roche, R.A., Nichols, D., Simpson, D.M. (1997). Knowledge, behavior, and fears concerning breast and cervical cancer among older low-income Mexican-American women. American Journal of Preventive Medicine, 13, 137-142.

62 Mayo, R.M., Ureda, J.R., & Parker, V.G. (2001). Importance of fatalism in understanding mammography screening in rural elderly women. Journal of Women and Aging, 13, 57-72.

63 Shankar, S., Selvin, E., & Alberg, A.J. (2002). Perceptions of cancer in an African-American community: a focus group report. Ethnicity and Disease, 12, 276-283.

64 Bailey, E. J. (1987). Sociocultural factors and health care-seeking behavior among Detroit Afro-Americans. Journal of the National Medical Association, 79, 389-392.

65 Weinrich, S., Coker, A. L., Weinrich, M., Eleazer, G. P., & Greene, F. L. (1995). Predictors of Pap smear screening in socioeconomically disadvantaged elderly women. Journal of the American Geriatric Society, 43, 267-70.

66 Schwartz, M.D., Hughes, C., Roth, J., Main, D., Peshkin, B.N., Isaacs, C., Kavanagh, C., Lerman, C. (2000). Spiritual faith and genetic testing decisions among high-risk breast cancer probands. Cancer, Epidemiology, Biomarkers and Prevention, 9, 381-385.

67 Hughes, C., Lerman, C., & Lustbader, E. (1996). Ethnic differences in risk perception among women at increased risk for breast cancer. Breast Cancer Research and Treatment, 40, 25-35.

68 Diefenbach, M.A., Miller, S.M., & Daly, M.B. (1999). Specific worry about breast cancer predicts mammography use in women at risk for breast and ovarian cancer. Health Psychology, 18, 532-536.

69 McCaul, K. D., Schroeder, D. M., & Reid, P. A. (1996). Breast cancer worry and screening: some prospective data. Health Psychology, 15, 430-433.

70 Lerman, C., Caputo, C., & Brody, D. (1990). Factors associated with inadequate cervical cancer screening among lower income primary care patients. Journal of the American Board of Family Practice, 3, 151-156.

71 Lerman, C., Daly, M., Sands, C., Balshem, A., Lustbader, E., Heggan, T., Goldstein, L., James, J., & Engstrom, P. (1993). Mammography adherence and psychological distress among women at risk for breast cancer. Journal of the National Cancer Institute, 85, 1074-1080.

72 Adams, M.L., Becker, H., & Colbert, A. (2001). African-American women's perceptions of mammography screening. Journal of the National Black Nurses Association, 12, 44-48.

73 Tessaro, I., Eng, E., & Smith, J. (1994). Breast cancer screening in older African-American women: qualitative research findings. American Journal of Health Promotion, 8, 286-292.

74 Epstein, S.A., Lin, T.H., Audrain, J., Stefanek, M., Rimer, B., & Lerman, C. (1997). Psychosomatics, 38, 253-261.

75 Lerman, C., Daly, M., Masny, A., & Balshem, A. (1994). Attitudes about genetic testing for breast-ovarian cancer susceptibility. Journal of Clinical Oncology, 12, 843-850.

76 Durfy, S. J., Bowen, D. J., McTiernan, A., Sporleder, J., & Burke, W. (1999). Attitudes and interest in genetic testing for breast and ovarian cancer susceptibility in diverse groups of women in western Washington. Cancer Epidemiology, Biomarkers, and Prevention, 8, 369-375.

77 Miller, S. M. (1996). Monitoring and blunting of threatening information: Cognitive interference and facilitation in the coping process. In G. Sarason (Ed.), Cognitive interference: Theories, methods, and findings. NJ: Lawrence Erlbaum.

78 Steptoe, A. & O'Sullivan, S.J. (1986). Monitoring and blunting coping styles in women prior to surgery. British Journal of Clinical Psychology, 25, 143-144.

79 Lerman, C., Schwartz, M. D., Miller, S. M., Daly, M., Sands, C., & Rimer, B. K. (1996). A randomized trial of breast cancer risk counseling: Interacting effects of counseling, educational level, and coping style. Journal of the National Cancer Institute, 87, 286-292.

80 Miller, S.M., Roussi, P., Altman, D., Helm, W., & Steinberg, A. (1994). Effects of coping style on psychological reactions to colposcopy among low-income minority women. Journal of Reproductive Medicine, 39, 711-718.

81 Tercyak, K., Hughes, C., Main, D., Snyder, C., Lynch, J., Lynch, H., & Lerman, C. (2001). Parental communication of BRCA1/2 genetic test results to children. Patient Education & Counseling, 42(3), 213-224.

82 Wardle, F., Collins, W., Pernet, A., Whitehead, M., Bourne, T., & Campbell, S. (1993). Psychological impact of screening for familial ovarian cancer. Journal of the National Cancer Institute, 85, 653-657.

83 Sherman, K.A., Miller, S.M., Rodoletz, M., Driscoll, J., Daly, M., Godwin, A., & Babb, J. (2002). Paper on: The role of monitoring and anticipated *BRCA1/2* carrier status on family communications intentions and plans among women with a hereditary pattern. The 23rd Annual Meeting of the Society of Behavioral Medicine , Washington, DC.

84 Patenaude, A.F., Guttmacher, A.E., & Collins, F.S. (2002). Genetic testing and psychology: new roles, new responsibilities. American Psychologist, April, 271-282.

85 Eng, E. (1993). Save our Sisters Project. Cancer, 72, 1071-7.

86 Sheinfeld Gorin, S (1997). Outcomes of social support for women survivors of breast cancer (pp. 278-91). In E. Mullen & JL Magnabosco (Eds.) Outcomes Measurement in the Human Services: Cross-Cutting Issues and Methods. Washington, DC: NASW Press.

87 Gotay, C.C. & Wilson, M.E. (1998). Social support and cancer screening in African Amreican, Hispanic, and Native American women. Cancer Practice, 6, 31-37.

88 Stokols, D (1992). Establishing and maintaining healthy environments: Toward a social ecology of health promotion. American Psychologist, 47 (1), 6-22.

89 House, JS & Landis, KR, & Umberson, D (1988). Social relationships and health. Science, 241, 540-45

90 House, J.S., Kahn, R.I. (1985). Measures and concepts of social supports. In: S. Cohen, S.L. Symens (Eds.) Social Support and Health. Orlando: Academic Press, 83-108.

91 Fox, SA, Murata, PJ & Stein, JA (1991). The impact of physician compliance on screening mammography by women. Annals of Behavioral Medicine, 151, 50-56.

92. Lane, DS & Brug, MA (1990). Breast cancer screening: Changing physician practices and specialty variation. New York State Journal of Medicine, 90, 288-92.

93. Ashford, A., Gemson, D., Sheinfeld Gorin, S., Bloch, S., Lantigua, R., Ahsan, H., & Neugut, AI. (2000). Cancer Screening and Prevention Practices of Inner City Physicians. American Journal of Preventive Medicine, 19, 59-62.

94 Mandelblatt, J.S., Yabroff, K.R. (1999). Effectiveness of interventions designed to increase mammography use: a meta-analysis of provider-targeted strategies. Cancer Epidemiology, Biomarkers, and Prevention, 8, 759-67.

95 Gemson, DH, Elinson, J, Messeri, P (1988). Differences in physicians' prevention practice for white and minority patients. Journal of Community Health, 4, 5-26.

96 Stefanek, ME (1993). Psychosocial aspects of breast cancer. Current Opinion in Oncology, 5, 996-1000.

97 Burg, MM & Seeman, TE (1994). Families and health: The negative side of social ties. Ann Behav Med, 16 (2), 109-115.

98 Burnett, C.B., Steakley, C.S., Tefft, M.C. (1995). Barriers to breast and cervical cancer screening in underserved women of the District of Columbia. Oncology Nursing Forum, 22, 1551-7.

99 Mickey, R.M., Durski, J., Worden, J.K., Dangelis, N.L. (1995). Breast cancer screening and associated factors for low-income African-American women. Preventive Medicine, 24, 167-76.

100 Kang, SH & Bloom, JR (1993). Screening among older black Americans. Journal of teh National Cancer Institute, 85, 737-42.

101 Gorin, S.S., & Jacobson, J. (2001). Diet and breast cancer surveillance behaviors among Harlem women. Annals of the New York Academy of Sciences, 952, 153-160.

102 Croyle, R., & Lerman, C. (1999). Risk communication in genetic testing for cancer susceptibility. Journal of the National Cancer Institute Monograms, 25, 59-66.

103 Kash, K., M, Lerman, C, Massie & Brown. (1993). Psychosocial issues in women at genetic risk for breast cancer. In I. J. H. e. al., Psycho-Oncology V: Psychosocial Factors in Cancer Risk and Survival. NY: Memorial Sloan-Kettering Cancer Center.

104 Eng, E. (1993). The Save Our Sisters Project. A social network strategy for reaching rural black women. Cancer, 72, 1071-1077.

105 Kang, S. H. & Bloom, J. R. (1993). Social support and cancer screening among older black Americans. Journal of the National Cancer Institute, 85, 737-742.

γ-Synuclein is over-expressed in breast and ovarian cancers and promotes tumor cell survival by inhibiting stress-induced apoptosis

Z. Pan[1], W. Bruening[1], B.I. Giasson[2], V.M. Lee[2], A.K. Godwin[1]
1) Fox Chase Cancer Center, Philadelphia, PA; 2) U. of Penn. School of Medicine.

Synucleins are a family of small, highly conserved proteins predominantly expressed in neurons. Although the normal functions of the synucleins are not entirely understood, α- and γ-synuclein have been implicated in the pathogenesis of several neurodegenerative diseases and cancer, respectively. Previous studies have shown that γ-synuclein, a candidate proto-oncogene also known as *BCSG1* or persyn, is dramatically up-regulated in the vast majority of late-stage breast (>70%) and ovarian (>85%) cancers and that γ-synuclein over-expression can enhance tumorigenicity. To address the biological function of γ-synuclein and its role in pathogenesis of the breast and ovary, we ectopically over-expressed γ-synuclein in several cancer cell lines. We found that γ-synuclein may promote tumor cell growth by protecting tumor cells from undergoing mitochondria involved caspase-9/3 apoptosis under adverse conditions. We have shown that γ-synuclein is associated with several mitogen-activated kinase (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1) Over-expression of γ-synuclein leads to constitutive activation of ERK1/2 and down-regulation of JNK1 in response to a host of environmental stress signals, include UV light, heat shock, sodium arsenate, and nitric oxide (NO). We have also found that γ-synuclein expressing cells are more resistant to the chemotherapeutic drug paclitaxel as compared to the parental cells. Activation of JNK by paclitaxel was blocked by γ-synuclein and the number of apoptotic cells induced by paclitaxel was significantly lower in cells that over-expressed γ-synuclein. This resistance could be partially obliterated when ERK activity was inhibited using a MEK1/2 inhibitor. Taken together, our data indicate that γ-synuclein is likely to be involved in the pathogenesis of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain anti-cancer drugs. Because of its high frequency of expression in late-stage breast and ovarian cancers, γ-synuclein appears to be a promising target for cancer therapy.

Gamma-synuclein, a candidate oncogene, activates RAC and ERK and contributes to the metastatic spread of breast and ovarian cancer.

Z. Pan[1], W. Bruening[1], B.I. Giasson[2], V.M. Lee[2], A.K. Godwin[1].
1) Fox Chase Cancer Center, Philadelphia, PA; 2) U. of Penn. School of Medicine.

The synucleins (α, β, γ, synoretin) are a family of small cytoplasmic proteins that are predominantly expressed in neurons. Although α-synuclein is known to play an important role in neurodegenerative diseases, the function of synucleins is unknown. We have previously reported that one member of the family, γ-synuclein, is expressed in the majority (>85%) of late-stage breast and ovarian carcinomas, but it is not expressed in normal mammary and ovarian epithelium. Therefore, we hypothesize that γ-synuclein may be a proto-oncogene and that abnormal expression of this protein may contribute to the progression of breast and ovarian cancer. In support of this hypothesis, we have observed that exogenous expression of γ-synuclein in tumor cells elicits a phenotype similar to that induced by activation of the Rho/Rac/CDC42 pathway, i.e., altering the appearance of focal adhesions and stress fibers, and enhancing motility and invasion. Strikingly, levels of activated Rac (GTP-bound) are constitutively elevated in ovarian tumor cells that overexpress γ-synuclein. Synuclein proteins also exhibit a low homology to the 14-3-3 family of cytoplasmic chaperone proteins. 14-3-3 proteins help regulate different signal transduction pathways by directly binding to various protein kinases and bringing them into close proximity with substrate and regulatory proteins. We have demonstrated a novel interaction of g-synuclein with the MAPKs, ERK1/2 and JNK1, in a single complex. Overexpression of γ-synuclein leads to increased ERK activation but does not activate JNK. As activated ERK1/2 specifically localize to focal adhesions, and ERK1/2 has been shown to enhance migration, these results raise the possibility that γ-synuclein may enhance the metastatic potential of tumors through the activation of Rac within the Rho signaling pathway based on protein interactions at focal adhesions. We hypothesize that γ-synuclein contributes to tumorigenesis by promoting cell motility as a result of altering the Rho and ERK1/2 signaling pathways and that g-synuclein may be an important therapeutic target.

Identification of ovarian cancer-associated genes using a HOSE cell transformation model

Andrey Frolov, Zhong-Zong Pan, Dominique Broccoli, Lisa Vanderveer, Nelly Auersperg[1], Henry Lynch[2], Mary Daly, Thomas Hamilton, and Andrew K. Godwin
Fox Chase Cancer Center
[1] University of British Columbia, Vancouver, British Columbia
[2] Creighton University School of Medicine, Omaha, NE

The diseases that are commonly referred to as ovarian cancer in the vast majority of cases develop from the malignant transformation of a single cell type, the surface epithelium. However, the biological mechanisms leading to transformation remain unclear. To identify genetic and biological determinants of ovarian cancer, we have developed an *in vitro* model for ovarian cancer. We have initiated primary human ovarian surface epithelial (HOSE) cell cultures and have derived HOSE cell lines that have undergone immortalization and spontaneous transformation *in vitro* and can form tumors *in vivo*. We have found that during immortalization, HOSE cells can use different pathways for telomere length maintenance. Maintenance of telomeric repeats is required for immortalization and is commonly associated with activity of telomerase. However, a number of tumors and tumor cell lines do not contain telomerase activity and circumvent the telomere length dependent limitation on cell division by a mechanism referred to as Alternative Lengthening of Telomeres (ALT). The mechanism(s) leading to ALT remains unknown. We have found that up to 30% of advanced stage ovarian adenocarcinomas lack telomerase activity. We have also observed that the majority of our HOSE cell cultures appear to use the ALT pathway for telomere maintenance, thereby providing an *in vitro* model to characterize the underlying basis of telomerase-dependent and independent ovarian tumorigenesis.

We have utilized Suppression Subtractive Hybridization (SSH) and cDNA microarray approaches to identify genes that are differentially expressed upon both immortalization and malignant transformation. One such gene, γ-synuclein, is a member of a family of small cytoplasmic proteins (i.e., α-, β-, γ-synuclein, and synoretin) that are predominantly expressed in neurons. The functions of the synucleins are not entirely understood, but they have been implicated in the pathogenesis of several neurodegenerative diseases. We have found that γ-synuclein, is expressed in the majority (>85%) of late-stage ovarian carcinomas, but it is not expressed in normal ovarian surface epithelium (Bruening, *et al.*, 2000). Therefore, we hypothesize that γ-synuclein may be a proto-oncogene and that abnormal expression of this protein in its oncogenic form may contribute to the progression and spread of ovarian cancer. In support of this hypothesis, we have observed that when γ-synuclein is exogenously expressed in cell lines derived from ovarian tumors, the cells become highly motile as observed by time-lapse photography and invasive as determined using a Boyden chamber assay. We further hypothesize that expression of γ-synuclein may be promoting this phenotype in part by modulating the Rho/Rac/CDC42 signal transduction pathway. Strikingly, levels of activated Rac (GTP-bound) are constitutively elevated in ovarian tumor cells that overexpress γ-synuclein.

Synuclein proteins also exhibit a weak homology to the 14-3-3 family of cytoplasmic chaperone proteins. The 14-3-3 family of proteins helps regulate many different signal transduction pathways, and is thought to act by directly binding to various protein kinases and bringing them into close proximity with substrate and regulatory proteins. We have recently demonstrated a novel interaction of γ-synuclein with a mitogen-activated kinase (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and that overexpression of γ-synuclein leads to increased ERK activation. As activated ERK1/2 specifically localize to focal adhesions, and ERK1/2 has been shown to enhance migration, these results raise the possibility that γ-synuclein may enhance the metastatic potential of

tumors through the activation of Rac within the Rho signaling pathway based on protein interactions at focal adhesions. Based on our results we hypothesize that γ-synuclein contributes to tumorigenesis by promoting cell motility as a result of altering the Rho and ERK1/2 signaling pathways and that γ-synuclein may not only be a biomarker for ovarian cancer, but a therapeutic target.

γ-Synuclein Promotes Cancer Cell Survival and Inhibits Stress- and Chemotherapy Drug-Induced Apoptosis by Modulating MAPK Pathways*

Zhong-Zong Pan‡, Wendy Bruening‡,§, Benoit I. Giasson¶, Virginia M-Y. Lee¶, and Andrew K. Godwin‡****

‡ Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111

§ Present address: Health Technology Assessment Information Service, Plymouth Meeting, PA 19462

¶ Center for Neurodegenerative Diseases, University of Pennsylvania School of Medicine, 3600 Spruce Street, Philadelphia, PA 19104

* This work was supported in part by the Eileen Stein-Jacoby Fund, the Ovarian Cancer Research Fund, a concept grant from the Department of Defense (DAMD17-01-1-0522), a grant from the NIH (Ovarian Cancer SPORE P50 CA83638), and by an appropriation from the Commonwealth of Pennsylvania. Z.Z.P. was supported by a fellowship from the Department of Defense (Institutional Breast Cancer Training Program grant DAMD17-00-1-0249), W.B. was supported by a fellowship from the National Institutes of Health; B.I.G. was supported by a fellowship from the Human Frontiers Foundation.

** To whom correspondence should be addressed: Dept. of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Rm. W322, Philadelphia, PA 19111. Tel.: 215-728-2205; Fax: 215-728-2741; E-mail: A_Godwin@fccc.edu.

1 The abbreviations used are: MAPK, mitogen-activated kinase; BCSG1, breast cancer-specific gene 1; ERK, extracellular signal-regulated protein kinase; JNK, c-JUN N-terminal kinase; MEK, MAP kinase kinase; NACP, the precursor of NAC (non-A component of AD amyloid); LBs, Lewy bodies; AD, Alzheimer's disease; DLB, dementia with LBs.

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

SUMMARY

Synucleins are a family of highly conserved small proteins predominantly expressed in neurons. Of the synucleins, α-synuclein is the best characterized because of its potential significance in neurodegenerative diseases. Recently we and others have found that γ-synuclein is dramatically up-regulated in the vast majority of late-stage breast and ovarian cancers and that γ-synuclein over-expression can enhance tumorigenicity. In the current study, we have found that γ-synuclein is associated with two major mitogen-activated kinases (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1) and have shown that over-expression of γ-synuclein leads to constitutive activation of ERK1/2 and down-regulation of JNK1 in response to a host of environmental stress signals including UV, arsenate, and heat shock. We also tested the effects of γ-synuclein on apoptosis and activation of JNK and ERK in response to several chemotherapy drugs. We have found that γ-synuclein expressing cells are significantly more resistant to the chemotherapeutic drugs paclitaxel and vinblastine as compared to the parental cells. The resistance to paclitaxel can be partially obliterated when ERK activity is inhibited using a MEK1/2 inhibitor. Activation of JNK and its downstream caspase-3 by paclitaxel or vinblastine is significantly down-regulated in γ-synuclein expressing cells, indicating that paclitaxel or vinblastine activated apoptosis pathway is blocked by γ-synuclein. In contrast to paclitaxel and vinblastine, etoposide does not activate JNK and γ-synuclein over-expression has no apparent effect on this drug-induced apoptosis. Taken together, our data indicate that oncogenic activation of γ-synuclein contributes to the development of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain chemotherapeutic drugs.

INTRODUCTION

Breast carcinoma is the second leading cause of cancer related deaths in women of the western world. In the United States alone over 180,000 new cases are diagnosed annually and more than 40,000 women die from this disease each year (1). Epithelial ovarian cancer continues to be the leading cause of death from gynecologic malignancies in the United States (1,2). One woman in 70 in the U.S. will develop ovarian cancer in her lifetime, and one woman in 100 will die of this disease. Breast and ovarian cancer etiology are multifactorial, involving environmental factors, hormones, genetic susceptibility, and genetic changes during progression. Both cancers are a heterogeneous group of tumors with no unifying molecular alteration yet identified. A certain number of breast and ovarian cancer cases (~5 to 10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (3)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic cancers is still poorly defined.

In an effort to identify other genes involved in the development and/or progression of breast and ovarian cancer, we and others used differential gene expression approaches and have found that γ-synuclein, initially termed breast cancer-specific gene 1 (BCSG1), is up-regulated in the majority of late-stage breast (4,5) and ovarian cancer [Bruening, 2000 #16; Pan and Godwin, unpublished data]. In addition, we showed that there was a correlation between γ-synuclein expression in breast ductal carcinomas and the staging of the cancer suggesting that γ-synuclein may be a potential marker for both late stage breast and ovarian cancer. Additional studies have revealed that γ-synuclein over-expression leads to increased invasiveness of breast tumor cells (6) and stimulated cell proliferation (7).

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

Synucleins are a family of small, highly soluble proteins that are predominantly expressed in neurons. The functions of the synucleins are not entirely understood. There are four known members: α-synuclein [also referred to as synelfin or non-Aβ component of Alzheimer's disease (AD) amyloid precursor protein (NACP)], and β-synuclein [also referred to as phosphoneuroprotein 14 (PNP-14)] are neuronal proteins primarily expressed in brain and are predominantly found at axonal terminals. γ-Synuclein [also known as persyn] is predominantly expressed in certain regions of the peripheral nervous system, such as dorsal root ganglia and trigeminal ganglia. Synoretin, the newest member of the synuclein family is expressed at high levels in the retina and at lower levels in the brain (8,9). The synuclein proteins contain several repeated domains that display variations of a KTKEGV consensus sequence. The β-synuclein protein contains five of these domains, whereas the α- and γ-synucleins have six. Interestingly, the third domain of each protein is completely conserved and this same type of domain is present in proteins of the Rho family (10). Another type of organization of the synuclein proteins that has been noted is an 11-residue repeat. This motif, repeated six to seven times in the amino-terminal portion of the protein, is reminiscent of the amphipathic α-helical domains of the apolipoproteins and suggests possible lipid binding properties (11).

The γ-synuclein gene maps to 10q23, is composed of five coding exons, and is transcribed into an ~1 kb mRNA (8). The human γ-synuclein is 127 amino acids long, and is 87.7% and 83.3% identical to the mouse and rat proteins, respectively. In addition, comparison of the amino acid sequences indicates that γ-synuclein is highly homologous to α-synuclein and β-synuclein except for the last 27 amino acids of γ-synuclein. Overall, γ-synuclein shares 54%, 56%, and 84% amino acid sequence identity with α-synuclein, β-synuclein, and synoretin, respectively

(8,9).

Among the synucleins, α-synuclein is the best characterized because of its significant role implicated in neurodegenerative diseases (12). Mutations in the α-synuclein gene have been identified in rare kindreds with Parkinson's disease (12-14) and intracytoplasmic aggregates comprised of α-synuclein fibrils are characteristic of several neurodegenerative diseases as exemplified by the intraneuronal Lewy bodies (LBs), neuroaxonal spheroids and dystrophic neurites (i.e. Lewy neurites) that are prominent in PD, LB variant of Alzheimer's disease (AD), and dementia with LBs (DLB) (14-19).

The normal physiological functions of synucleins are not well characterized. The N-terminal portion of α-synuclein (residues 1-61) shares 40% amino acid homology with members of the 14-3-3 protein family (20). The 14-3-3 family of proteins helps regulate many different signal transduction pathways, and is thought to act by directly binding to various protein kinases and bringing them into close proximity with substrate and regulatory proteins. 14-3-3 proteins bind to phospho-Ser residues critical for the functions of many kinases and phosphatases that are involved in diverse cell functions (21-24). Like the synucleins, 14-3-3 proteins are ubiquitously expressed in the brain and have been shoown to associate in a chaperone-like manner with PKC, BAD, ERK, and RAF-1 (16,21). α-Synuclein binds 14-3-3 as well as to PKC, BAD, ERK and the microtubule associated protein tau (25). In addition to shared regions of homology to 14-3-3, α-synuclein, as well as β- and γ-synuclein also appears to act as a protein chaperone, at least *in vitro*, by disrupting protein aggregation (26).

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

To help further unravel the function of γ-synuclein and establish its role in the oncogenesis of breast and ovarian cancer, we searched for proteins that could interact with γ-synuclein and identified the MAP kinases ERK1/2 and JNK1. In this study we provide evidence that γ-synuclein contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways. In addition, we observed that paclitaxel- or vinblastine-induced cell death is protected by γ-synuclein indicating that chemotherapeutic drugs that take effect through activating the JNK apoptosis pathway may not be effective for cancer with high γ-synuclein expression.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies – Paclitaxel, vinblastine, and etoposide were purchased from Sigma (St. Louis, MO). The MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI). Anti-ERK1, anti-ERK2, anti-JNK1 antibodies and normal IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2 and anti-PARP antibodies were obtained from Cell Signaling (Beverly, MA). The mouse antibody Syn303 was raised to recombinant human α-synuclein, but recognizes α-, β-, and γ-synuclein. γ-2 is a rabbit polyclonal antibody raised to recombinant human γ-synuclein that specifically recognizes human γ-synuclein (27). Mouse monoclonal antibodies Syn204 and Syn207 were raised against human α- and β-synucleins, respectively.

Cell culture and transfection - Ovarian cancer cell lines A2780 and OVCAR5 were maintained in 10% FBS DMEM and 10% FBS RPMI 1640, respectively. HEK 293, human embryonic kidney cells, were maintained in 10% FBS DMEM supplemented with Na-pyruvate and non-essential amino acids. To create the CMV plasmid for establishing stable cell lines over-expressing human α-, β- or γ-synuclein, human cDNAs were amplified by PCR and subcloned into pcDNA3 (Invitrogen). GenePorter Transfection Reagent (GTS Inc, San Diego, CA) was used for transfection and stable cell lines were selected by G418 (Gibco BRL). Expression of human α-, β- or γ-synuclein was confirmed by immuno-blotting with Syn204, Syn207, or γ-2 antibody, respectively.

Cell treatment with UV and other stress signals or chemotherapeutic drugs - For UV treatment, cells at 70-80% confluence were washed once with PBS before UV irradiation (254

nm, 20 J/m^2). Complete medium was added to the plates after treatment. The intensity of the UV light source was measured with a BLAK-RAY meter (UVP, Inc., San Gabriel, CA) prior to each experiment. Heat-shock was carried at 42°C for 15 min. 50 μM Na-arsenite was used to treat the cells for 6 hr. Paclitaxel, vinblastine, etoposide, and U0126 were dissolved in DMSO and cells were treated with various concentrations of the drugs as indicated in each experiment.

Co-Immunoprecipitation - Cells at 70-80% confluence were washed twice with ice-cold D-PBS before scraping on ice with Lysis Buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM Na-pyrophosphate; 1 mM Na-β-glycerophosphate; 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100, and 1 tablet of protease inhibitor cocktail (Roche, Indianapolis, IN) per 40 ml lysis buffer]. Cellular debris was removed by centrifugation (14,000 x g for 15 min at 4°C) and precleared with protein G-agarose (Gibco BRL, Rockville, MD). Protein concentrations were determined with Bio-Rad DC protein assay reagents. Syn303 (3 μl ascites) or control IgG (3 μg) were pre-incubated in 500 μl of PBS with 50 μl of protein-G agarose overnight at 4°C, and washed twice with PBS before incubation with 300 μg of total cellular lysate for 4 hr at 4°C. The beads were washed 4 x with the lysis buffer, resuspended in 50 μl 2 x SDS sample buffer before boiling for 5 min. 10 μl immunoprecipitates were separated by SDS-PAGE electrophoresis on 4-20% linear gradient Tris-HCl ready gels (Bio-Rad).

Immuno-blotting and data quantification - Proteins separated on SDS-PAGE gels were transferred onto Immobilon-P PVDF membrane (Millipore, Bedford, MA). The primary antibodies were diluted 1:1000, and the HRP-conjugated second antibodies were diluted

1:10,000 (Amersham, Piscataway, NJ). NEN Renaissance Enhanced Luminol Reagents (Boston, MA) were used as substrates for detection. For re-use of the same membrane with another primary antibody, Restore Western Blot Stripping buffer (Pierce, Rocklord, IL) was used to strip the membrane. The results of immuno-blotting were quantitated using the NIH Image for the integrated density of each band.

JNK-kinase activity assay - The kinase activity of JNK was measured using the SAPK/JNK assay kit (Cell Signaling Technologies). Briefly, 250 µl cell lysate (1 µg/µl protein) was incubated with 2 µg c-JUN fusion protein beads (in 20 µl) overnight at 4°C. After washing, the proteins on the beads were incubated in the kinase reaction buffer supplemented with 100 µM ATP for 30 min at 30°C. To measure the JNK activity, the phosphorylated c-JUN was detected by SDS-PAGE and immunoblotting with the specific antibody (Cell Signaling Technologies).

Cell viability assay - Cell viability was determined by Trypan blue exclusion assay and/or WST-1 assay. For Trypan blue assay, cells were stained with 0.2% Trypan blue for 2-5 min. The number of viable cells (non-stained) and dead cells (stained) were counted under microscope using a cell hemocytometer. For WST-1 assay, cells under different culture conditions were incubated with WST-1 (Roche) for 4 hr. Cleavage of WST-1 to formazan was monitored at 450 nm using a microplate reader.

Caspase activity assay - Colorimetric CaspACE assay (Promega) was used to detect the caspase-3 activity. Briefly, pNA released from the substrate Ac-DEVD-pNA by caspase-3 in the cell lysate was monitored at 405 nm using a microplate reader.

Statistical analysis – Where indicated, a two-tailed student t-test was used to test for significance.

RESULTS

Expression of synuclein in tumor cell lines - We have previously reported that γ-synuclein is highly expressed in the vast majority of late-stage breast and ovarian tumors (4), suggesting a potentially important role for γ-synuclein in the development of these diseases. To help unravel the function of γ-synuclein, we established several *in vitro* models. The ovarian tumor cell lines A2780 and OVCAR5, which express low levels of γ-synuclein as well as kidney HEK 293 cells which do not express detectable levels of γ-synuclein were transfected with CMV-γ-synuclein or with vector alone and were selected with G418. Resistant colonies were screened by Western blotting for stable expression of γ-synuclein protein and positive colonies were pooled into A2780gam, OVCAR5gam, and 293gam cell lines (**Fig. 1**). Cell lines stably expressing α-synuclein and β-synuclein were also derived from A2780 cells as described for the γ-synuclein expressing lines (**Fig. 1**). Like A2780gam, OVCAR5gam, 293gam cells, there were no obvious alterations in cell doubling time of the A2780alpha or A2780beta cell lines (data not shown).

γ-Synuclein interacts with ERK and JNK MAP kinases in cancer cells - γ-Synuclein has recently been reported to bind directly to the ERK2 kinase (28). Therefore, we evaluated whether γ-synuclein could also interact with the ERK kinases as well as other MAPKs. By co-immunoprecipitation approaches, we were able to demonstrate a novel association of γ-synucleins with ERK1/2 and JNK1 kinase, but not with the p38 kinase (**Fig. 2**). We also confirmed that α-synuclein is associated with ERK1/2 as well with JNK1 (**Fig. 2**), which is consistent with the recently studies using neuro2a, a neuronal cell line (29). These data indicate that γ- and α-synuclein can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

Elevated activity of ERK in cells over-expressing γ-synuclein - We next evaluated whether these protein interactions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ-synuclein, the activated ERK1/2 was increased 2-3 fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (**Fig. 3**). In contrast, α- and β-synucleins appeared to have little or no effect on the activity of ERK1/2 (**Fig. 3A**) although α-synuclein was also found to be associated with ERK (as described above and shown in **Fig. 2**) in A2780 cells. In HEK 293 cells, the basal level of ERK activation is undetectable and γ-synuclein over-expression does not increase its activation level (**Fig. 3B**). Structural analysis indicate that γ-synuclein does not contain any kinase domain, suggesting that the activation of ERK is mediated by other kinase. Since MEK1/2 is required for the activation of ERK1/2 in response to many mitogens, we determined whether MEK1/2 is still required for γ-synuclein mediated activation of ERK1/2. When cells over-expressing γ-synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (**Fig. 4A**). We further studied the relation of γ-synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ-synuclein and ERK1/2 was still present (**Fig. 4B**). These data indicate that γ-synuclein may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ-synuclein.

Down-regulation of JNK activation by γ-synuclein in response to different stress signals - JNK is activated by stress signals including UV which leads to mitochondria mediated apoptosis (30). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ-synuclein was over-expressed or not (**Fig. 5**). JNK was highly

activated in the parental cells when treated with UV (**Fig. 5**). In cells over-expressing γ-synuclein, the activation of JNK was almost completely blocked in A2780/gam cells ($p < 0.05$) and was down-regulated by approximately 50% in OVCAR5/gam cells when treated with UV (**Fig. 5**) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ-synuclein specific since over-expression of α- and β-synucleins appeared to have little or no effect on the activation of JNK by UV (**Fig. 5**, and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific, or it could be the high endogenous γ-synuclein expression in OVCAR5 cells. Similarly, the activation of JNK by sodium arsenate was blocked to different extents by γ-synuclein in 293/gam, OVCAR5/gam, and A2780/gam cells (**Fig. 6**). Collectively, these data indicate that stress-induced activation of JNK can be blocked by γ-synuclein over-expression in a variety of cell lines.

γ-Synuclein may protect paclitaxel (Taxol) induced cell death by regulating JNK and ERK activities - Based on the data presented above, we hypothesized that γ-synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated γ-synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, cell survival and cell death are regulated by the counter-balance between the survival factors and the apoptotic signaling pathways. Since UV treatment also activates the cell survival pathways ERK [(31), and data not shown] and PI3K-AKT (32,33), the initiation of the mitochondria associated caspase pathway may be blocked by the activation of ERK or AKT

in A2780 cells. In support of these findings we did not observe the cleavage of the caspase-3 substrate PARP in A2780 cells when treated with UV (data not shown).

We next evaluated the survival of γ-synuclein over-expressing cells in response to Taxol, a commonly used chemotherapeutic drug. In addition to its role in affecting microtubule assembly, Taxol is known to lead to apoptosis via the mitochondria by activating the JNK signaling pathway and Taxol-induced apoptosis can be enhanced by MEK inhibition (34-36). In A2780 cells, Taxol did not affect the basal level activity of ERK or the activation of ERK by γ-synuclein (**Fig. 7A**). To test the effect of γ-synuclein on cell survival, cells were treated with Taxol for varying lengths of time. At 48hr after treatment, 45-60% of A2780 cells had died, while only about 7-15% of A2780/gam cells were dead indicating that Taxol induced cell death can be rescued by γ-synuclein over-expression (**Fig. 7B**). When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced by ~25% in A2780 cells but was nearly doubled in A2780/gam cells (**Fig. 7B**). These data suggest that enhanced cell survival in γ-synuclein over-expressing cells is partially mediated by activation of ERK.

To determine whether the protective role of γ-synuclein on cell survival is also mediated through down-regulating JNK associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (34,36), JNK was activated in A2780 cells when treated with 30 μM Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol ($p < 0.05$) (**Fig. 8A**). In the parental A2780 cells, caspase-3 was highly

activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in γ-synuclein over-expressing ovarian cancer cells ($p < 0.05$) (**Fig. 8B**). These data indicate that Taxol activated JNK mediated caspase apoptotic pathway was significantly attenuated in cells over-expressing γ-synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by γ-synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

γ-Synuclein over-expression leads to protection from vinblastine but not etoposide induced cell death – To demonstrate whether the effects of γ-synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents; Taxol binds to microtubule polymers while vinblastine binds to monomers and dimers. When treated with vinblastine (either 0.1, 1.0, or 10 μM for 30 hr), cell death in A2780/gam cells was significantly lower ($p < 0.05$ for all the three concentrations tested) as compared to the parental cells (**Fig. 9A**). Consistent with other studies using a variety of tumor cell lines (36-39), vinblastine strongly activate JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by γ-synuclein over-expression (**Fig. 9B**). Furthermore, we observe that treatment with vinblastine results in a two-fold increase in phosphorylated ERK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (**Fig. 9B**). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (**Fig. 9A & C**). These data indicate that suppression of vinblastine-induced cell death by γ-synuclein may be mediated by inhibition of JNK activation.

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (37,40-42). When treated with 1, 10, or 100 μM of etoposide for 56 hrs, there was no significant difference in cell survival between A2780 and A2780/gam cells (**Fig. 10A**). As might be predicted, JNK was not activated in response to etoposide treatment (**Fig. 10B**). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down-regulated within 30 min of treatment with 10 or 100 μM of etoposide (**Fig. 10C**). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and γ-synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.

DISCUSSION

Synucleins are highly soluble proteins and their biological and biochemical functions are not entirely understood. Previous studies have suggested that they may be involved in neuron development and function (43). The involvement of γ-synuclein in human neoplastic diseases came to light when γ-synuclein was isolated from a human breast tumor cDNA library and was shown to be over-expressed in infiltrating ductal carcinomas (4,5) and ovarian cancer (4). Additional studies have suggested that γ-synuclein may be involved in enhancing cell motility and metastasis, in breast (6) and ovarian (Bruening and Godwin, unpublished data) cancer cells as analyzed both *in vitro* and in nude mouse models *in vivo*. We have found that oncogenic activation of γ-synuclein is independent of gene mutations or gene amplification. We have recently reported that hypomethylation of the γ-synuclein gene CpG island promotes its aberrant expression in breast and ovarian carcinomas (44,45). In the present studies, we showed that γ-synuclein over-expression activates the survival factor ERK1/2 and blocks the activation of JNK. Activation of JNK can initiate the mitochondria involved caspase apoptosis pathway (30). Therefore, we propose that γ-synuclein, in its oncogenic form (over-expressed) contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways (**Fig. 11**). In addition, we observed that Taxol- and vinblastine-induced cell death is protected by γ-synuclein indicating that anti-cancer drugs that take effect through activating the JNK/caspase apoptosis pathway may not be effective for cancers with high γ-synuclein protein levels.

The effects of γ-synuclein on ERK1/2 and JNK signaling in ovarian cancer cells appears to be specific for γ-synuclein since over-expression of the α- and β-synucleins in A2780 cells had

little or no effect on these MAPKs (**Figs. 3 & 5**). Even though α- and β-synucleins are expressed in a significant fraction of ovarian tumors (4), we did not observe a discernable phenotype associated with over-expression in cultured cells. The reason for these differences are not readily apparent. However, we have recently observed that the subcellular localization of γ-synuclein in tumor cells may be altered. Previous studies have shown that γ-synuclein is diffusely distributed in the cytoplasm of peripheral neurons, although it is also expressed in the brain (46). We have found in tumor cells which over-express the wild-type γ-synuclein that the protein tends to accumulate in the nuclei (Pan and Godwin, unpublished data). This may also help explain why only a portion of ERK1/2 and JNK co-immunoprecipates with γ-synuclein (**Fig. 2**), since the cytoplasmic levels are low in A2780 cells. Furthermore, the amino acid sequences of α- and β-synucleins are more closely related to each other than γ-synuclein (8,9) and the γ-synuclein protein is the least conserved of the synuclein proteins (8,9). Therefore, it is possible that the conserved sequences of α, β, and γ-synuclein may be involved in the interaction with ERK1/2 and that the non-conserved regions (predominantly the C-terminus) may contribute to activation of ERK in a cell type specific manner. Therefore, the effects that we are observing may be both cell type and isoform specific. Additional studies will be needed to better resolve these differences.

Even though we have made some significant observations in this study, the biochemical function of γ-synuclein remains largely unknown. Many of our approaches have come from the study of α-synuclein. α-Synuclein, the most extensively studied synuclein, is the major component of Lewy bodies in sporadic PD, dementia with LBs (DLB), and a subtype of Alzheimer's disease known as the LB variant of Alzheimer's disease (19,47,48). Mutations in

the α-synuclein gene have also been linked to familial Parkinson's disease (49-51). Based on homology to 14-3-3 protein and *in vitro* assay, synucleins are hypothesized to be proteins with chaperone properties (20,26). α-Synuclein has been shown to be associated with many of the proteins that interact with 14-3-3 proteins, including PKC, BAD, and ERK (16,21,25). α-Synuclein has also been shown to associate with ERK2 (28) and may reduce the phosphorylation of MAPKs in neurons (29), while synoretin can activate Elk1 pathway when transfected in HEK 293 cells (9). We have demonstrated that in ovarian cancer cells that both γ- and α-synucleins can associate with ERK1/2 (**Fig. 2**). However, only γ-synuclein but not α-synuclein lead to constitutive activation of ERK indicating that the function of α-synuclein might be different in cancer cells versus neuronal cells. Studies are underway to determine whether this constitutive activation is dependent on the C-terminal sequences which are unique to γ-synuclein.

The second prominent observation we made in this study was that ectopic expression of γ-synuclein resulted in enhanced resistance to the chemotherapeutic drugs, Taxol and vinblastine (**Figs. 7-9**). In contrast, when these same cells were treated with etoposide, a DNA damage agent, γ-synuclein over-expression did not enhanced cell survival (**Fig. 10**). There are two main apoptosis pathways initiated from the cell surface membrane, one is initiated by Fas and other death receptors, while the other is initiated by stress signals (30). JNK activation is an essential component of the latter apoptotic signaling (30,52). In addition, many chemotherapy drugs also take effect partly through activating the JNK signaling (34-36). Our data indicate that abnormal over-expression of γ-synuclein might be one of such mechanisms in breast and ovarian cancer that permits tumor cells to overcome the JNK activated apoptotic signaling (30). Both Taxol and vinblastine robustly induced JNK activity in A2780 cells, while the etoposide treatment did little

(**Figs. 8-10**). In γ-synuclein over-expressing cells, this induction by Taxol and vinblastine was suppressed 2- to 3-fold and cell survival was dramatically enhanced. Taken together, these results indicate that the cell death in ovarian cancer cells induced by Taxol, vinblastine, or other drugs that induce JNK, may be protected by γ-synuclein. Furthermore, it is interesting to note that only ~35% of breast tumors and ~50% of all epithelial ovarian tumors possess mutations in the *TP53* gene (53,54). As JNK activation in UV or Taxol treated cells is independent of DNA damage (31,55-57), it is interesting to speculate that over-expression of γ-synuclein may promote tumor cell survival in the presence of p53 wild-type cells. Since γ-synuclein is not expressed in normal breast and ovarian epithelial cells but is expressed in the majority of late-stage breast and ovarian cancers (4,5), it may be a very promising target to develop drugs for the therapies of these diseases.

Our results of γ-synuclein in cancer cells seem different from those of α-synuclein in neuronal cells. It remains to be determined whether these differences are caused by the different cellular context between cancer and neuronal cells, by different subcellular localization of the proteins in various normal and tumor cell types, or by innate difference in protein functions between γ- and α- synucleins. α-Synuclein over-expression in HEK 293 cells or A2780 cells does not induce apoptosis [Iwata, 2001 #101; Pan and Godwin, data not shown]. In neurons, mutant α-synuclein may accelerate apoptosis (58-61) while wild-type α-synuclein may induce or block apoptosis, depending on the path of apoptosis induction (29,58-60,62). In Parkinson's diseases, mitochondrial dysfunction and oxidative stress are believed to be the two main causes for neuronal death (63). The apoptotic role of wild-type α-synuclein in neuronal apoptosis may be secondary by disrupting mitochondria and causing oxidative stress (64). There is evidence

that the roles of synuclein in neurodegenerative diseases appear to be quite different. In contrast to the role of α-synuclein in neuronal degeneration, γ-synuclein does not cause neuronal apoptosis (65). γ- and β-Synucleins are not detected in Lewy bodies or Lewy neurites (8,14), and they are intrinsically less fibrillogenic than α-synuclein and cannot form mixed fibrils with α-synuclein (66). Therefore, it would be of interest to determine whether the normal protective role of γ-synuclein is lost in the neurons of PD and other neurodegenerative diseases. Overall, our studies provide new insight into the biological function of γ-synuclein and its role in the pathogenesis of the breast and ovary and offer a new therapeutic target for future treatment.

ACKNOWLEDGEMENTS

We would like to thank Drs. Alfred Knudson, Erica Golemis, Maureen Murphy and Anthony Yeung and Ms. Eryn Godwin for their valuable input and for the support from various FCCC Core Facilities.

FIGURE LEGENDS

Fig. 1. Establishment of cell lines that ectopically over-express γ-synuclein. Whole cell lysates from the parental cells or stably-transfected cells were analyzed by SDS-PAGE and immuno-blotting. Protein levels of γ-synuclein were determined by Western blotting with γ-2, a polyclonal antibody specific for γ-synuclein. Protein loading levels were evaluated by immuno-blotting with anti-α-actin antibody. The numbers beneath each band represent the densitometry units (A2780 was assigned the arbitrary unit 1.0 in the α-actin blot). γ-Synuclein protein levels were normalized to the protein levels of α-actin. The graph represents the average ± S.E. of at least three independent experiments, and representative blots are shown here. Molecular mass standards (in kilodaltons) are indicated to the left of each blot.

Fig. 2. Interaction between γ- and α-synucleins with ERK1/2 and JNK. Cell lysate from A2780, A2780/gam or A2780/alpha were immunoprecipitated with Syn303, NIg (normal IgG) or irrelevant antibodies (not shown) as described in the Experimental Procedures section. The proteins in the immunoprecipitates were identified by immunoblotting with antibodies against ERK1/2, JNK1, p38, and γ-2 (a polyclonal antibody specific for γ-synuclein). Molecular mass standards (in kilodaltons) are indicated on the left. Non-specific bands around the IgG heavy (**) and light (*) chains are indicated by asterisks.

Fig. 3. Activation of ERK in cells over-expressing γ-synuclein. A, ERK1/2 activation is enhanced in γ-synuclein over-expressing A2780 cells. Whole cell lysates (40 μg/lane) from A2780 cells (parent) or A2780 cells transfected with γ-, β-, or α-synuclein were separated by SDS-PAGE and blotted with appropriate antibodies. The levels of activated ERK or total

ERK1/2 were determined using an anti-phospho-ERK specific antibody or ERK1/2 antibodies, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band (the integrated densitometry reading number of A2780 or OVCAR5 was assigned to an arbitrary unit 1.0, and the other readings were normalized thereafter). The synucleins were evaluated by blotting with specific antibodies, i.e., γ-2 for γ-synuclein, Syn207 for β-synuclein, and Syn204 for α-synuclein, respectively. **B,** activation of ERK by γ-synuclein in OVCAR5 cells but not HEK 293 cells. Whole cell lysate (40 μg/lane) from parental or γ-synuclein over-expressing cells were separated and blotted as in **A**. Panels A and B are representative of at least three independent experiments with comparable results. **C,** fold increase of ERK activation. The data shown are the average ± S.E. of three independent experiments. Phosphorylated ERK was normalized to the protein level of total ERK. The basal levels of ERK phosphorylation in the parental A2780 or OVCAR5 cells were set as 1.0. (*) Represents significant difference compared to the parental cells ($p < 0.05$).

Fig. 4. Requirement of MEK1/2 for γ-synuclein enhanced ERK1/2 activation. A, A2780 and A2780/gam cells untreated or treated with the MEK1/2 inhibitor, U0126 (10 μM), were lysed and 30 μg of proteins were loaded into each lane. As in **Fig. 3**, anti- phospho-ERK1/2 specific antibody was used to detect activated ERK1/2; and the antibody against ERK1/2 was used to detect the protein level of total ERK1/2. **B,** the interaction between ERK and γ-synuclein is independent of the activation status of ERK1/2. A2780/gam cells in normal 10% FBS medium, 10% FBS medium with U0126 (10 μM), or serum-free medium were lysed and immunoprecipitated with Syn303 or control IgG. The proteins in the immunoprecipitates were detected with the antibodies against ERK1/2 or against human γ-synuclein (γ-2). The

autoradiogram shown is the representative of three independent experiments with comparable results.

Fig. 5. Inhibition of JNK activation by γ-synuclein in response to UV treatment. A2780, A2780/gam, A2780/beta, OVCAR5, OVCAR5/gam cells were un-treated or treated with UV (20 J/M^2) and cells were lysed at 30 min. JNK activities were analyzed by an immunocomplex kinase assay using GST-c-JUN as substrate. The phosphorylated GST-c-JUN by activated JNK was evaluated by immunoblotting with anti-phospho-c-JUN specific antibody (see Experimental Procedures for details). The protein levels of JNK and γ-synuclein were determined by immunoblotting with anti-JNK and γ-2 antibody, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. (*) Represents significant inhibition of JNK activation compared to that in the parental cells ($p < 0.05$).

Fig. 6. JNK activation is down-regulated by over-expression of γ-synuclein in response to sodium arsenate. HEK293, OVCAR5, and A2780 cells and their γ-synuclein over-expressing counterparts were treated with Na-arsenite (50 μM) for 6 hrs. Cells were lysed and the extracts assayed for JNK activity as described in the legends for **Fig. 5**. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are

representative of three independent experiments with comparable results.

Fig. 7. γ-Synuclein protects cells from paclitaxel (Taxol) induced cell death and is partially mediated by ERK activation. A, Taxol does not affect ERK activity or γ-synuclein mediated ERK activation. A2780 or A2780/gam cells pre-treated with or without U0126 (10 μM) were treated with Taxol (30 μM) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **B,** cell death induced by Taxol was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with Taxol for 48hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$).

Fig. 8. Taxol activated JNK and caspase-3 apoptotic pathway is blocked by γ-synuclein. A, down-regulation of JNK activation by Taxol in cells over-expressing γ-synuclein. Cell lysates from A2780 and A2780/gam cells treated with or without Taxol (30 μM) for 60 min were assayed for JNK activity (see the legend for **Fig. 5** for experimental details). The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of

total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. **B,** down-regulation of caspase-3 activity activated by Taxol in A2780/gam cells. A2780 and A2780/gam cells treated with Taxol (30 μM) for different time lengths were lysed, and approximately 20 μg protein were incubated with the caspase-3 substrate Ac-DEVD-pNA for 4hr at 37ÚC. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate reader. The graph represents the average ± S.E. of three independent experiments. In panels A and B, (*) represents significant difference compared to that in the parental cells ($p < 0.05$).

Fig. 9. Vinblastine induced cell death and activation of the MAPK pathways in γ-synuclein over-expressing cells. A, the cell death induced by vinblastine was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with vinblastine (0.1, 1, or 10 μM) for 30 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$). **B,** inhibition of JNK activation by γ-synuclein in response to vinblastine treatment. A2780 and A2780/gam cells were un-treated or treated with vinblastine (1μM, and 10μM) for 30 min and 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in the legend for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C,** the effect of vinblastine on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with vinblastine (1 μM and 10 μM) in the absence or presence of U0126 (10

μM) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

Fig. 10. Effects of γ-synuclein over-expression on etoposide induced cell death and activation of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express γ-synuclein. A2780 and A2780/gam cells treated with etoposide (1 μM, 10 μM, 100 μM) for 56 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. **B,** vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 μM, and 100 μM) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in **Fig. 5**. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C,** effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 μM and 100 μM) in the absence or presence of U0126 (10 μM) for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results.

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

Fig. 11. A proposed model illustrating how γ-synuclein promotes cell survival by modulating the ERK and JNK pathways.

REFERENCE

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. (1999) *CA Cancer J. Clin.* **49**(1), 8-31

2. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. (2000) *CA Cancer J Clin* **50**(1), 7-33.

3. Bove, B., Dunbrack, R., and Godwin, A. K. (2002) in *Breast Cancer: Prognosis, Treatment and Prevention.* (Pasqualini., J., ed), in press, Marcel Dekker Inc.

4. Bruening, W., Giasson, B. I., Klein-Szanto, A. J., Lee, V. M., Trojanowski, J. Q., and Godwin, A. K. (2000) *Cancer* **88**(9), 2154-2163.

5. Ji, H., Liu, Y. E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B. K., Rosen, C., and Shi, Y. E. (1997) *Cancer Res* **57**(4), 759-764.

6. Jia, T., Liu, Y. E., Liu, J., and Shi, Y. E. (1999) *Cancer Res* **59**(3), 742-747.

7. Liu, J., Spence, M. J., Zhang, Y. L., Jiang, Y., Liu, Y. E., and Shi, Y. E. (2000) *Breast Cancer Res Treat* **62**(2), 99-107.

8. Lavedan, C. (1998) *Genome Res* **8**(9), 871-880.

9. Surguchov, A., Surgucheva, I., Solessio, E., and Baehr, W. (1999) *Mol Cell Neurosci* **13**(2), 95-103.

10. Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) *J Neurosci* **8**(8), 2804-2815.

11. Clayton, D. F., and George, J. M. (1998) *Trends Neurosci.* **21**, 249-254

12. Mukaetova-Ladinska, E. B., Hurt, J., Jakes, R., Xuereb, J., Honer, W. G., and Wischik, C. M. (2000) *J Neuropathol Exp Neurol* **59**(5), 408-417.

13. Arima, K., Hirai, S., Sunohara, N., Aoto, K., Izumiyama, Y., Ueda, K., Ikeda, K., and

Kawai, M. (1999) *Brain Res* **843**(1-2), 53-61.

14. Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998) *Am J Pathol* **152**(4), 879-884.

15. Tu, P. H., Galvin, J. E., Baba, M., Giasson, B. I., Tomita, T., Leight, S., Nakajo, S., Iwatssubo, T., Trojanowski, J. Q., and Lee, V. M.-Y. (1998) *Annals Neurol.* **44,** 415-422

16. Arawaka, S., Saito, Y., Murayama, S., and Mori, H. (1998) *Neurology* **51,** 887-889

17. Arima, K., Ueda, K., Sunohara, N., Hirai, S., Izumiyama, Y., Tonozuka-Uehara, H., and Kawai, M. (1998) *Brain Res.* **808,** 93-100

18. Irizarry, M. C., Growdon, W., Gomez-Isla, T., Newell, K., George, J. M., Clayton, D. F., and Hyman, B. T. (1998) *J.Neuropathol.Exp.Neurol.* **57,** 334-337

19. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and M., G. (1998) *Proc.Natl.Acad.Sci.U.S.A* **95,** 6469-6473

20. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J., and Wolozin, B. (1999) *J Neurosci* **19**(14), 5782-5791.

21. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) *Annu Rev Pharmacol Toxicol* **40,** 617-647.

22. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84**(6), 889-897.

23. Yaffe, M. B., and Elia, A. E. (2001) *Curr Opin Cell Biol* **13**(2), 131-138.

24. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) *Cell* **91**(7), 961-971.

25. Tzivion, G., Luo, Z., and Avruch, J. (1998) *Nature* **394**(6688), 88-92.

26. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. (2000) *FEBS Lett.* **474,** 116-119

27. Giasson, B. I., Duda, J. E., Forman, M. S., Lee, V. M.-Y., and Trojanowski, J. Q. (2001) *Exp. Neurol* **172,** 354-362

28. Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001) *J Neurochem* **77**(1), 239-252.

29. Iwata, A., Maruyama, M., Kanazawa, I., and Nukina, N. (2001) *J Biol Chem* **276**(48), 45320-453229.

30. Davis, R. J. (2000) *Cell* **103**(2), 239-252.

31. Rosette, C., and Karin, M. (1996) *Science* **274**(5290), 1194-1197.

32. Krasilnikov, M., Adler, V., Fuchs, S. Y., Dong, Z., Haimovitz-Friedman, A., Herlyn, M., and Ronai, Z. (1999) *Mol Carcinog* **24**(1), 64-69.

33. Nomura, M., Kaji, A., Ma, W. Y., Zhong, S., Liu, G., Bowden, G. T., Miyamoto, K. I., and Dong, Z. (2001) *J Biol Chem* **276**(27), 25558-25567.

34. Lee, L. F., Li, G., Templeton, D. J., and Ting, J. P. (1998) *J Biol Chem* **273**(43), 28253-28260.

35. Mandlekar, S., Yu, R., Tan, T. H., and Kong, A. N. (2000) *Cancer Res* **60**(21), 5995-6000.

36. Wang, T. H., Popp, D. M., Wang, H. S., Saitoh, M., Mural, J. G., Henley, D. C., Ichijo, H., and Wimalasena, J. (1999) *J Biol Chem* **274**(12), 8208-8216.

37. Osborn, M. T., and Chambers, T. C. (1996) *J Biol Chem* **271**(48), 30950-5.

38. Stone, A. A., and Chambers, T. C. (2000) *Exp Cell Res* **254**(1), 110-119.

39. Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W. A., and Chambers, T. C. (2000) *J Biol Chem* **275**(39), 29980-29985.

40. Anderson, S. M., Reyland, M. E., Hunter, S., Deisher, L. M., Barzen, K. A., and Quissell,

D. O. (1999) *Cell Death Differ* **6**(5), 454-462.

41. Gibson, S., Widmann, C., and Johnson, G. L. (1999) *J Biol Chem* **274**(16), 10916-10922.

42. Jarvis, W. D., Johnson, C. R., Fornari, F. A., Park, J. S., Dent, P., and Grant, S. (1999) *J Pharmacol Exp Ther* **290**(3), 1384-1392.

43. Galvin, J. E., Lee, V. M., and Trojanowski, J. Q. (2001) *Arch Neurol* **58**(2), 186-190.

44. Gupta, A., Godwin, A. K., Vanderveer, L., Lu, A. P., and Liu, J. (2002) *Cancer Research*, submitted

45. Lu, A., Gupta, A., Li, C., Ahlborn, T. E., Ma, Y., Shi, E. Y., and Liu, J. (2001) *Oncogene* **20**(37), 5173-5185.

46. Buchman, V. L., Hunter, H. J., Pinon, L. G., Thompson, J., Privalova, E. M., Ninkina, N. N., and Davies, A. M. (1998) *J Neurosci* **18**(22), 9335-9341.

47. Goedert, M., Jakes, R., Crowther, R. A., Hasegawa, M., Smith, M. J., and Spillantini, M. G. (1998) *Biochem Soc Trans* **26**(3), 463-471.

48. Trojanowski, J. Q., and Lee, V. M. (1998) *Arch Neurol* **55**(2), 151-152.

49. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) *Science* **276**(5321), 2045-2047.

50. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L., and Riess, O. (1998) *Nat Genet* **18**(2), 106-108.

51. Papadimitriou, A., Veletza, V., Hadjigeorgiou, G. M., Patrikiou, A., Hirano, M., and Anastasopoulos, I. (1999) *Neurology* **52**(3), 651-654.

52. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288**(5467), 870-874.

53. Shahin, M. S., Hughes, J. H., Sood, A. K., and Buller, R. E. (2000) *Cancer* **89**(9), 2006-2017.

54. Thorlacius, S., Thorgilsson, B., Bjornsson, J., Tryggvadottir, L., Borresen, A. L., Ogmundsdottir, H. M., and Eyfjord, J. E. (1995) *Eur J Cancer* **31A**(11), 1856-1861.

55. Devary, Y., Rosette, C., DiDonato, J. A., and Karin, M. (1993) *Science* **261**(5127), 1442-1445.

56. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) *Cell* **71**(7), 1081-1091.

57. Wang, T. H., Wang, H. S., and Soong, Y. K. (2000) *Cancer* **88**(11), 2619-2628.

58. Lee, M., Hyun, D., Halliwell, B., and Jenner, P. (2001) *J Neurochem* **76**(4), 998-1009.

59. Tanaka, Y., Engelender, S., Igarashi, S., Rao, R. K., Wanner, T., Tanzi, R. E., Sawa, A., V, L. D., Dawson, T. M., and Ross, C. A. (2001) *Hum Mol Genet* **10**(9), 919-926.

60. Zhou, W., Hurlbert, M. S., Schaack, J., Prasad, K. N., and Freed, C. R. (2000) *Brain Res* **866**(1-2), 33-43.

61. Tabrizi, S. J., Orth, M., Wilkinson, J. M., Taanman, J. W., Warner, T. T., Cooper, J. M., and Schapira, A. H. (2000) *Hum Mol Genet* **9**(18), 2683-2689.

62. da Costa, C. A., Ancolio, K., and Checler, F. (2000) *J Biol Chem* **275**(31), 24065-24069.

63. Mizuno, Y., Yoshino, H., Ikebe, S., Hattori, N., Kobayashi, T., Shimoda-Matsubayashi, S., Matsumine, H., and Kondo, T. (1998) *Ann Neurol* **44**(3 Suppl 1), S99-109.

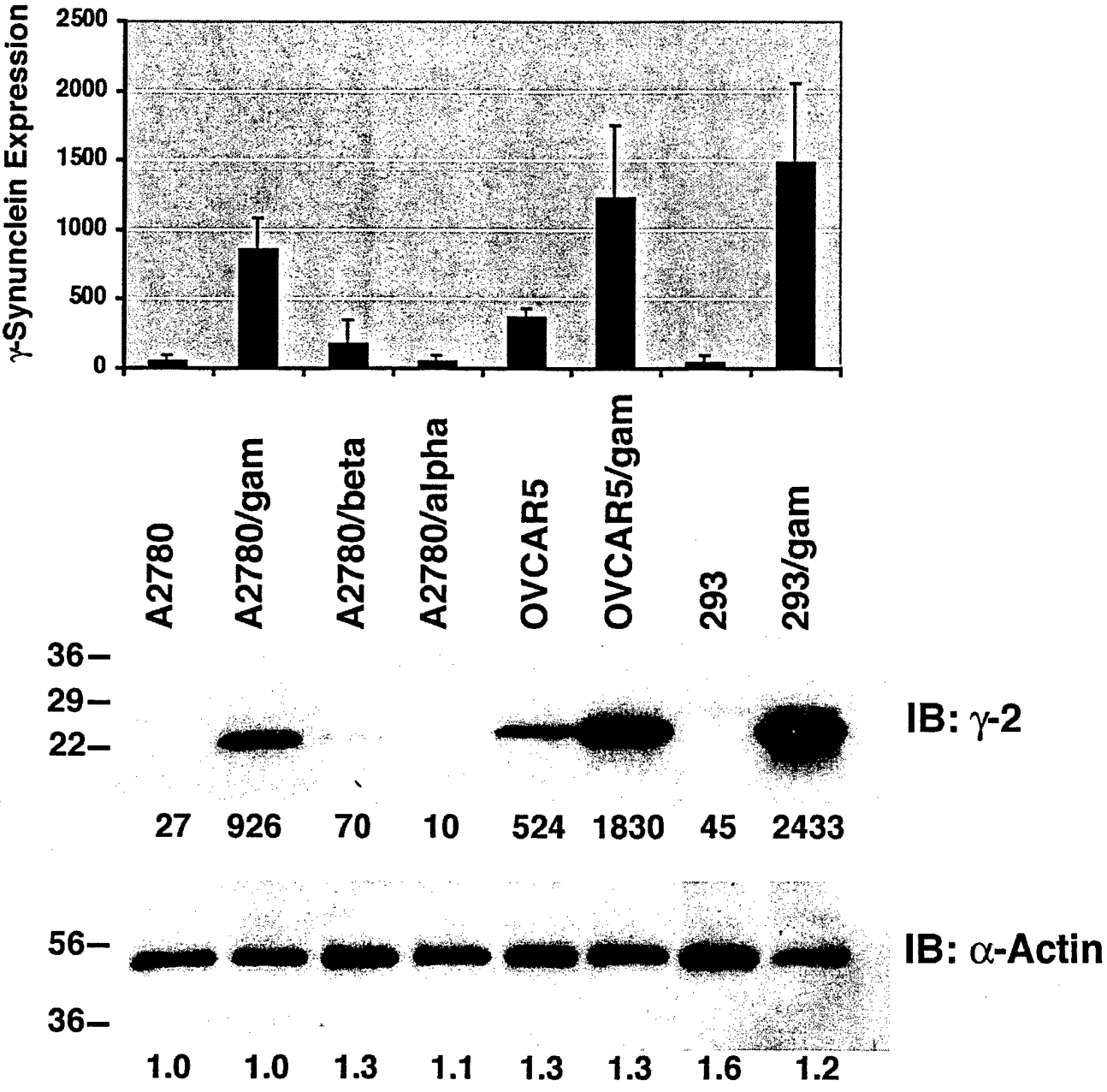
64. Hsu, L. J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., and Masliah, E. (2000) *Am J Pathol* **157**(2), 401-410.

65. Saha, A. R., Ninkina, N. N., Hanger, D. P., Anderton, B. H., Davies, A. M., and

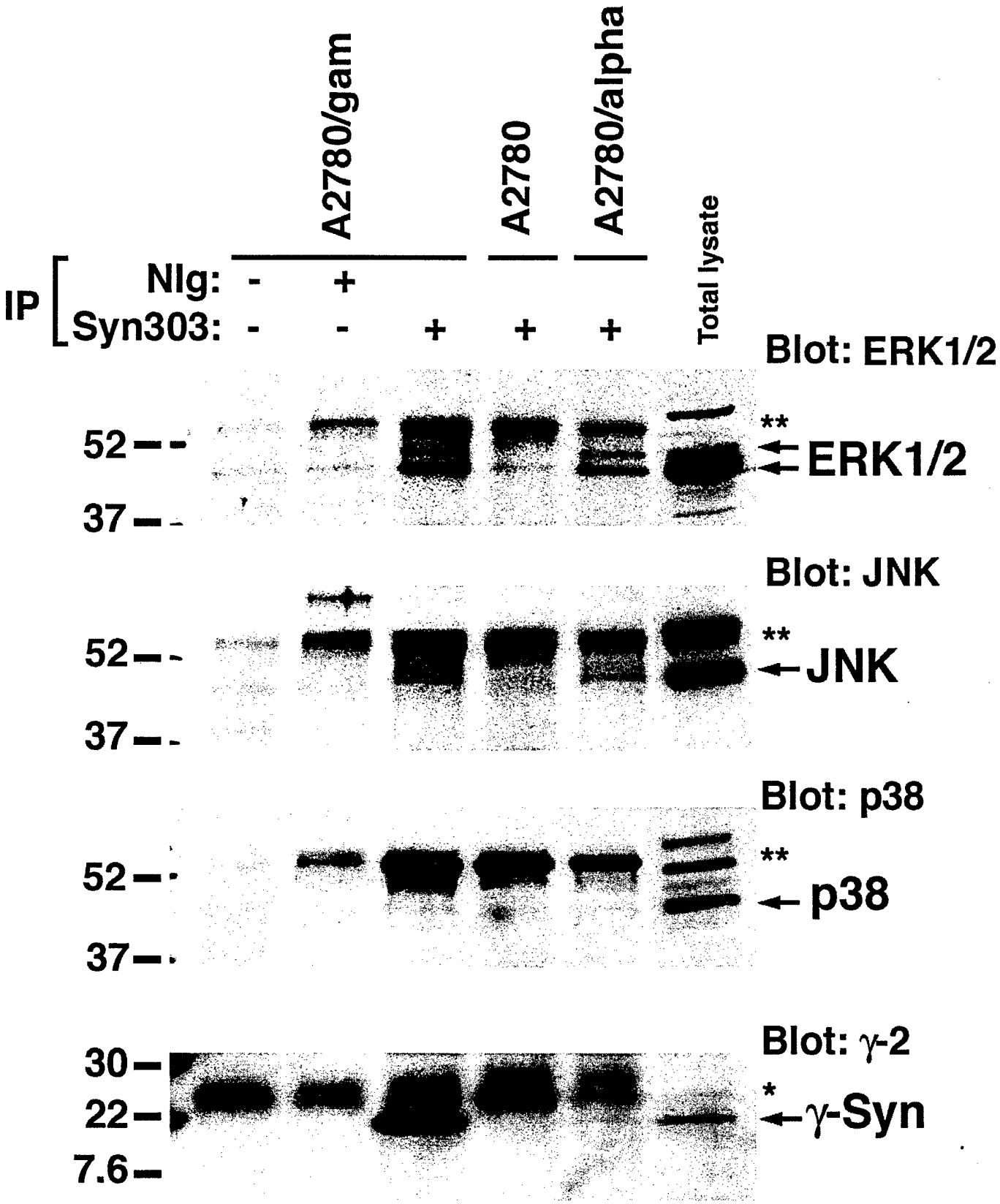
Buchman, V. L. (2000) *Eur J Neurosci* **12**(8), 3073-3077.

66. Biere, A. L., Wood, S. J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F. W., Jarosinski, M. A., Wu, G. M., Louis, J. C., Martin, F., Narhi, L. O., and Citron, M. (2000) *J Biol Chem* **275**(44), 34574-34579.

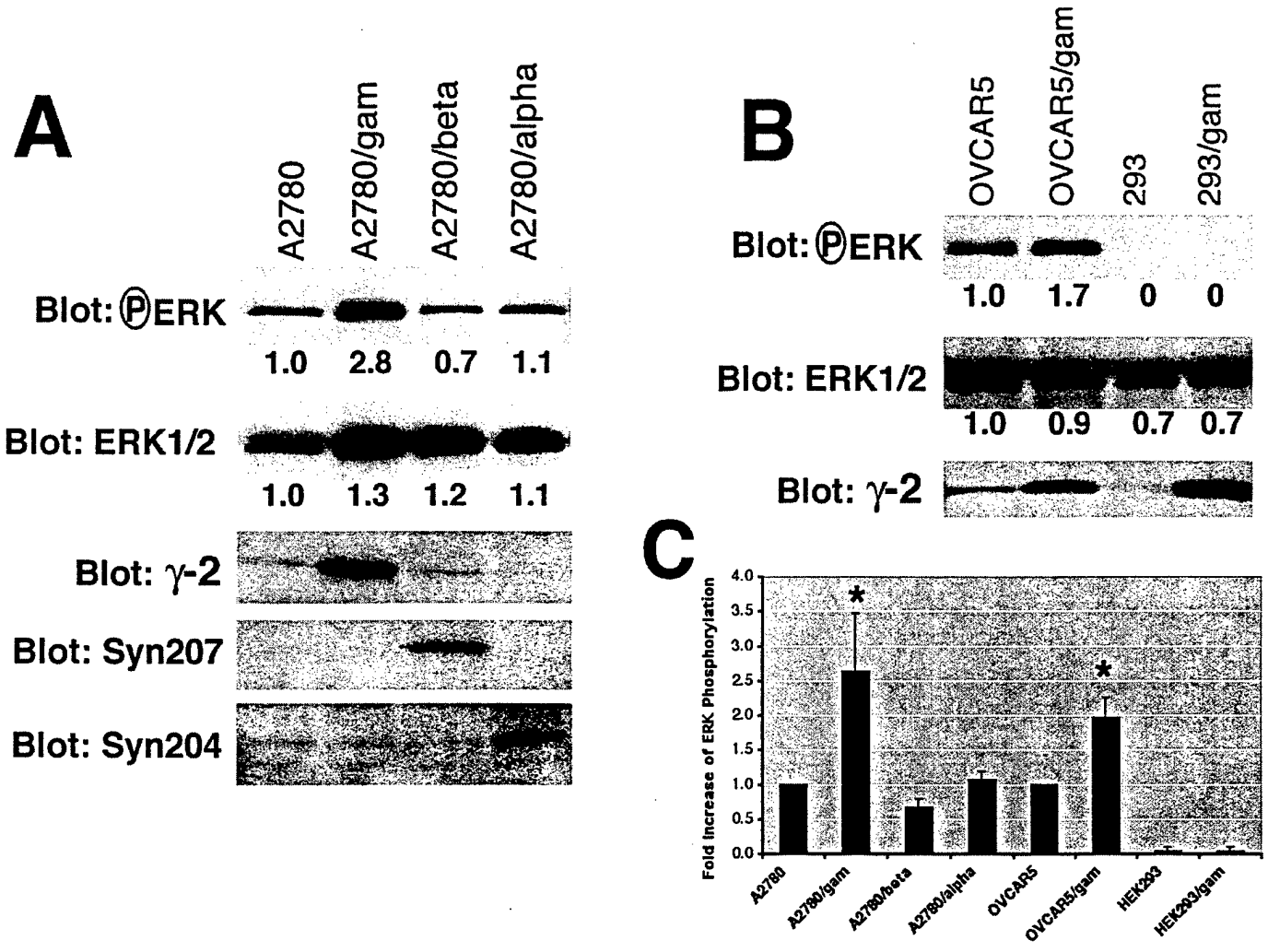
Pan et al. Fig.1



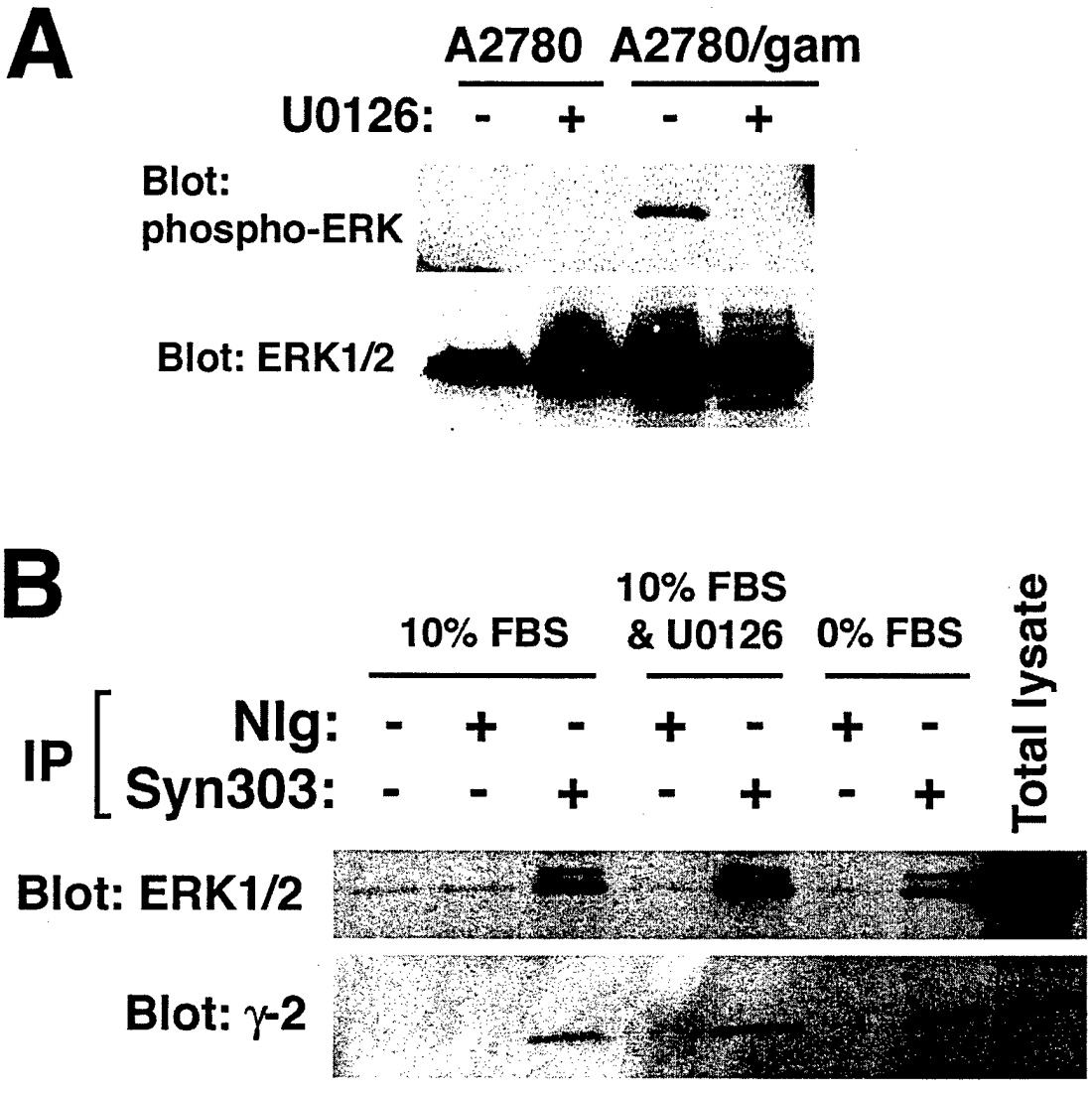
Pan et al. Fig.2



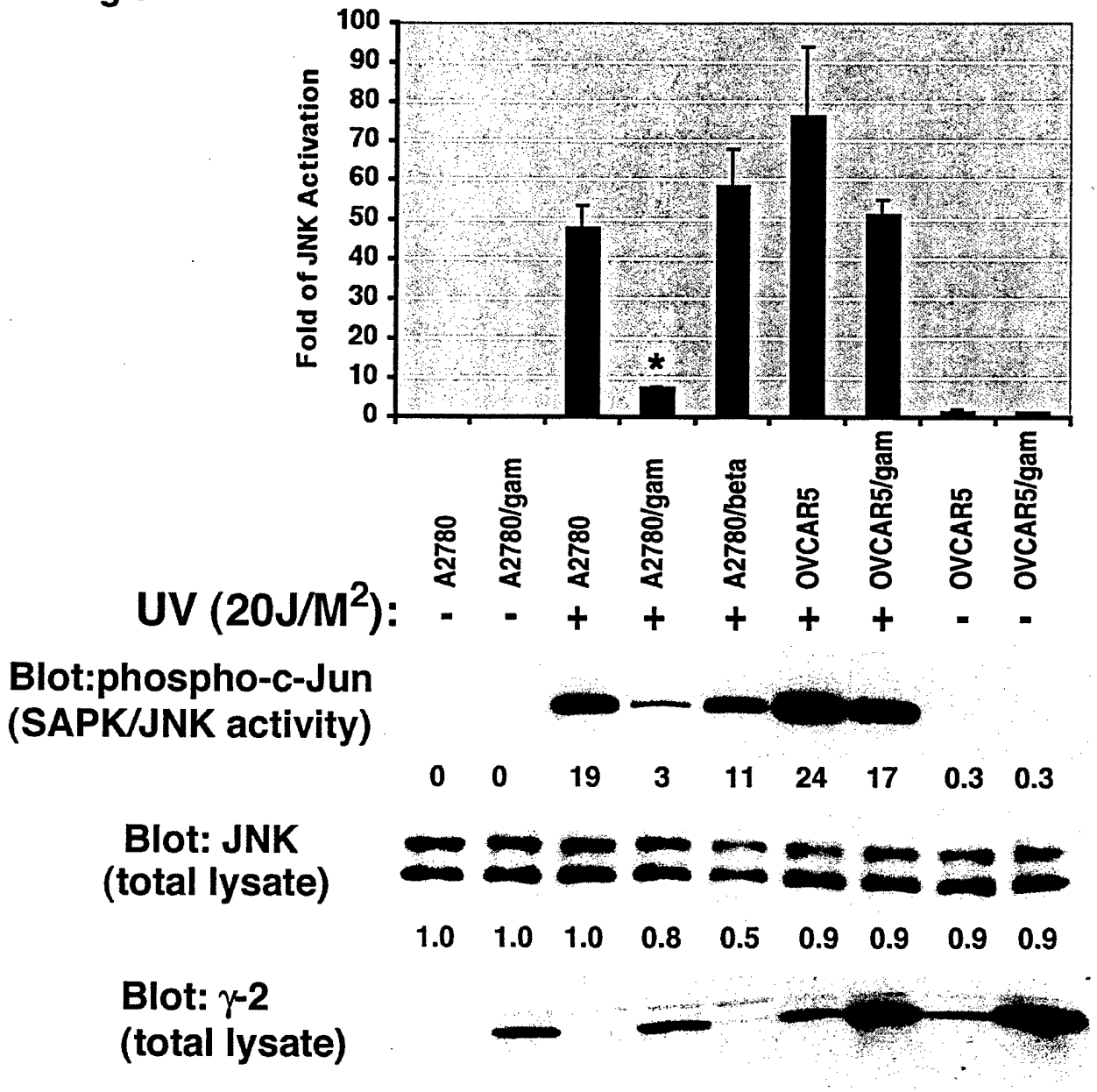
Pan et al. Fig.3



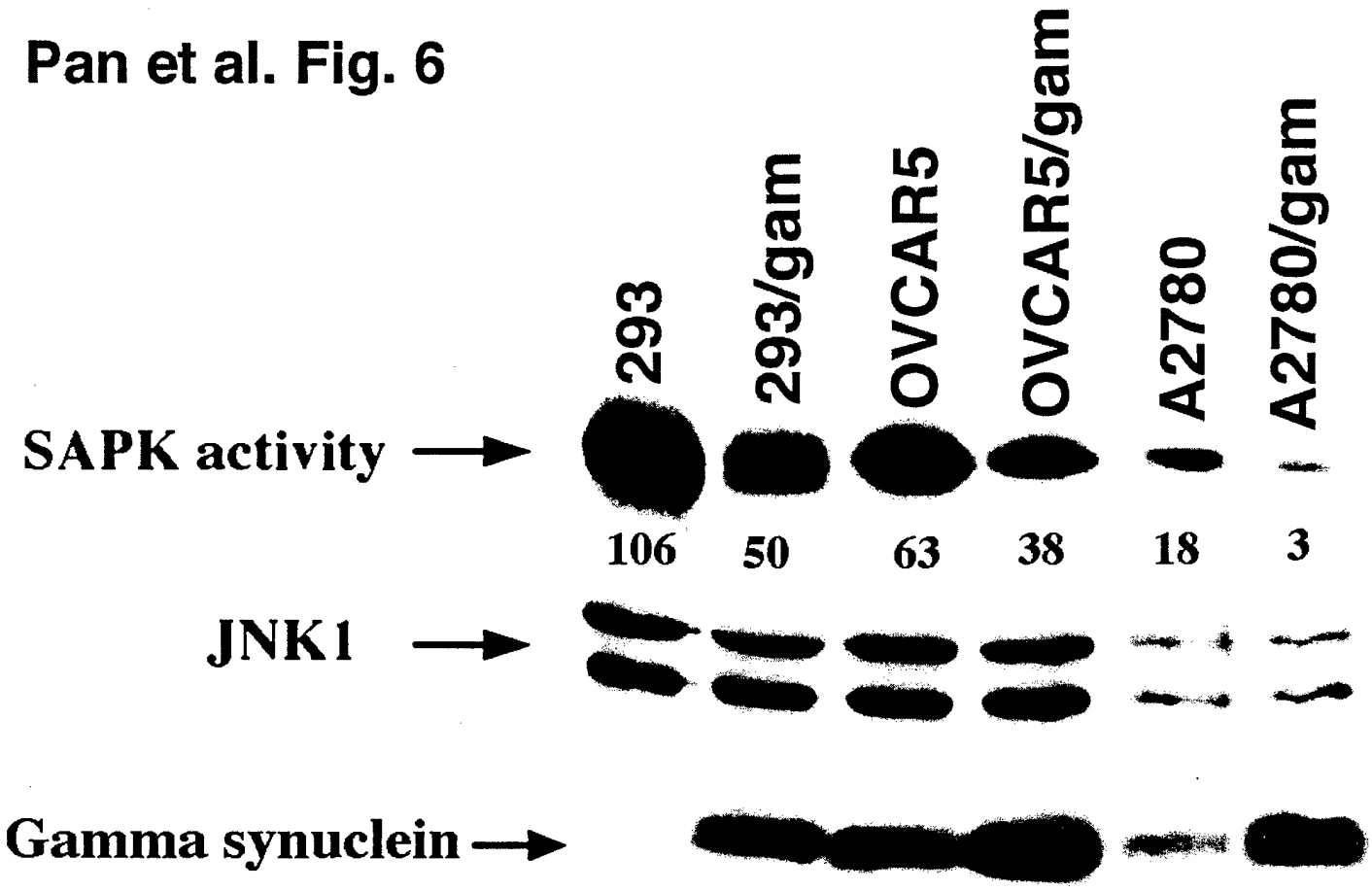
Pan et al. Fig.4



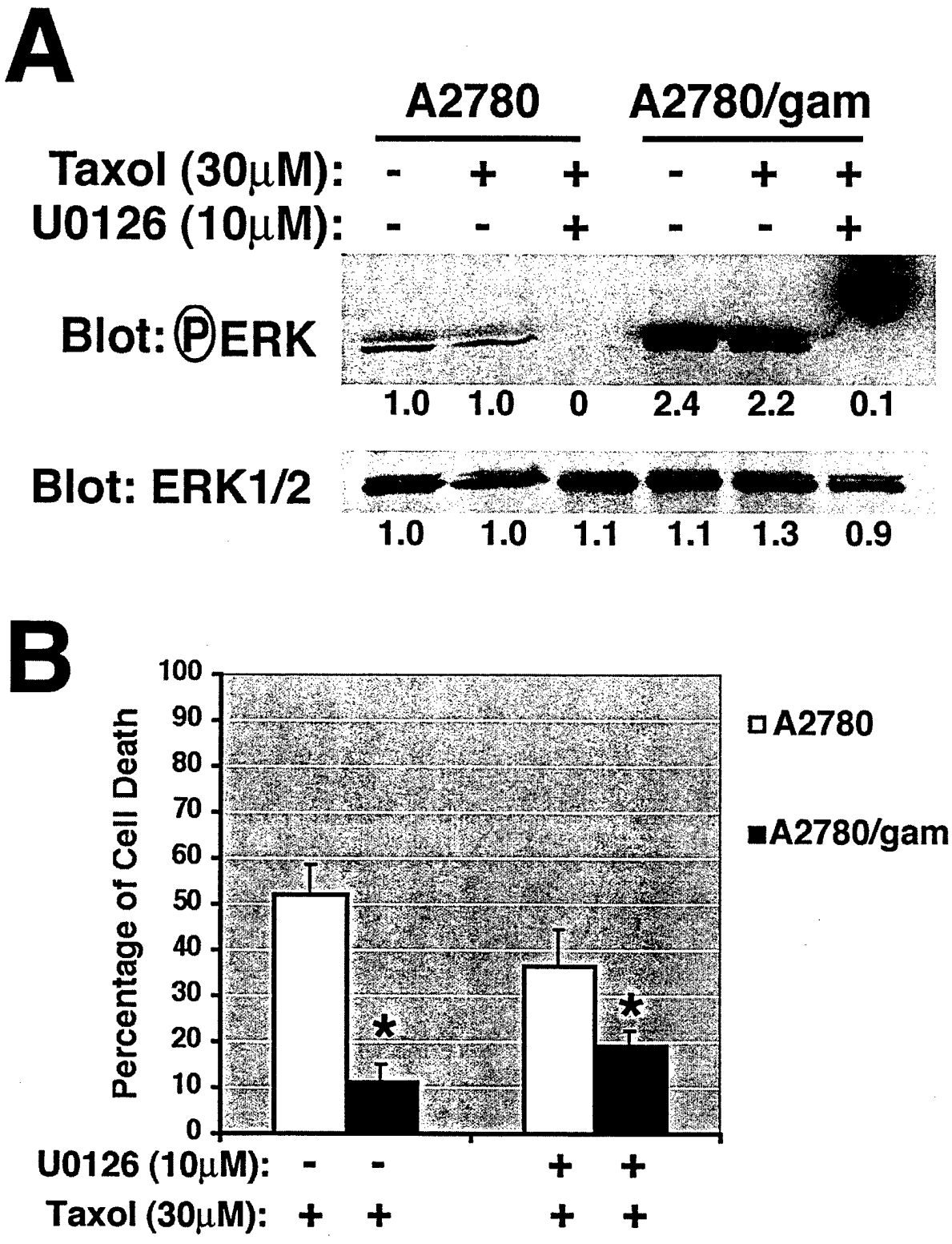
Pan et al. Fig.5



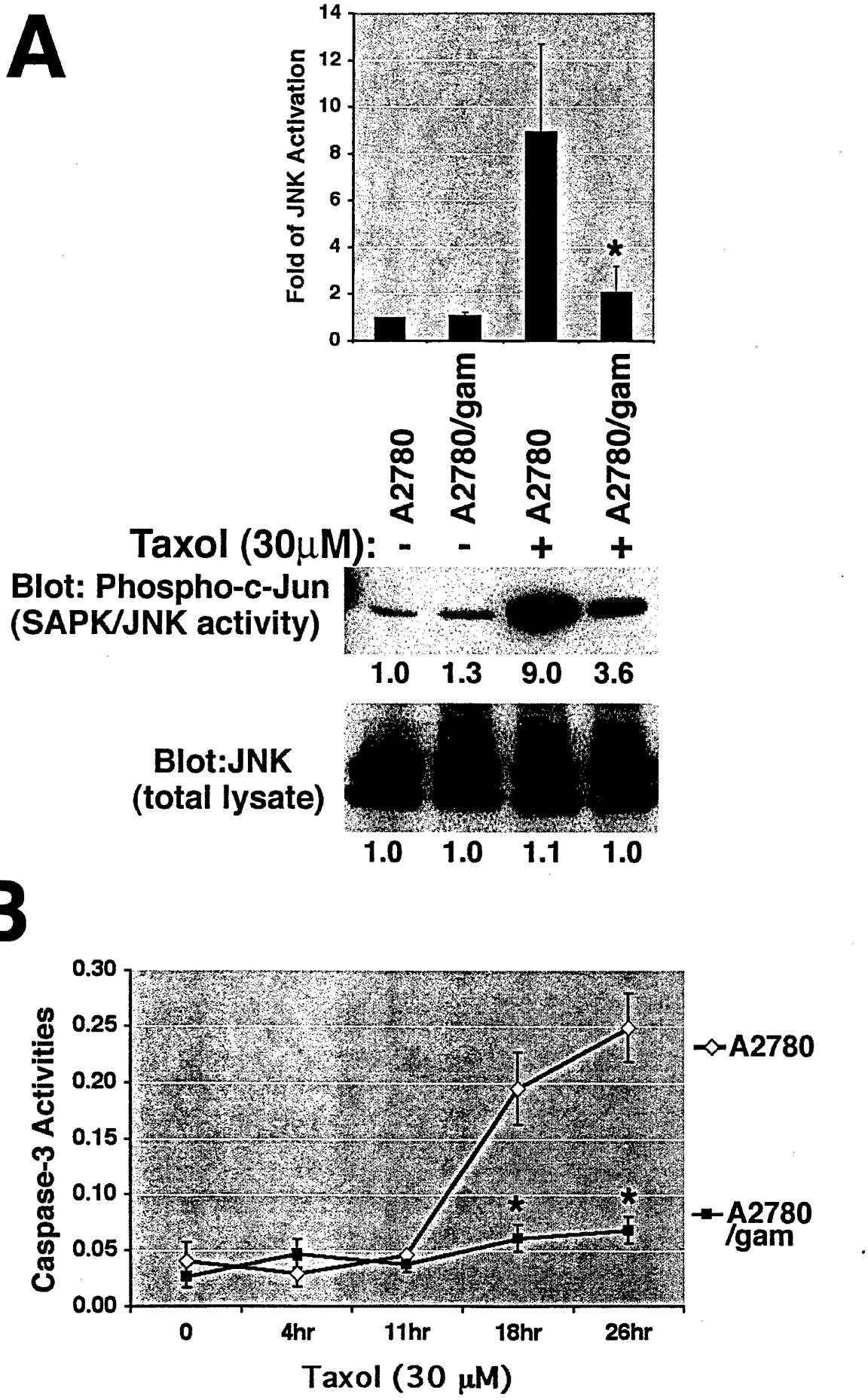
Pan et al. Fig. 6



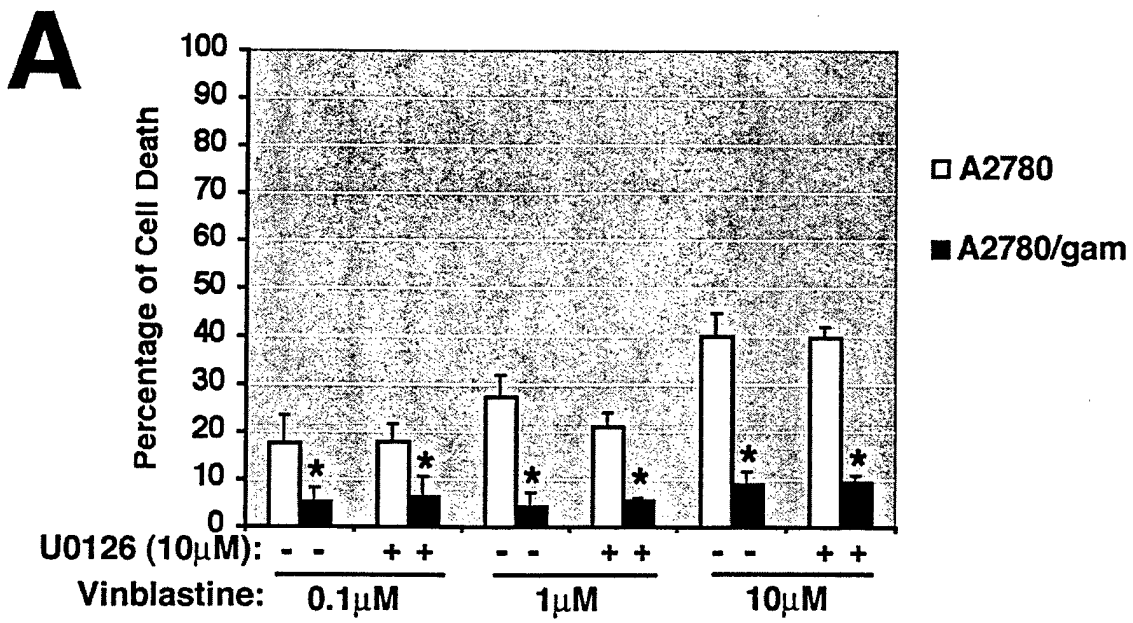
Pan et al. Fig.7

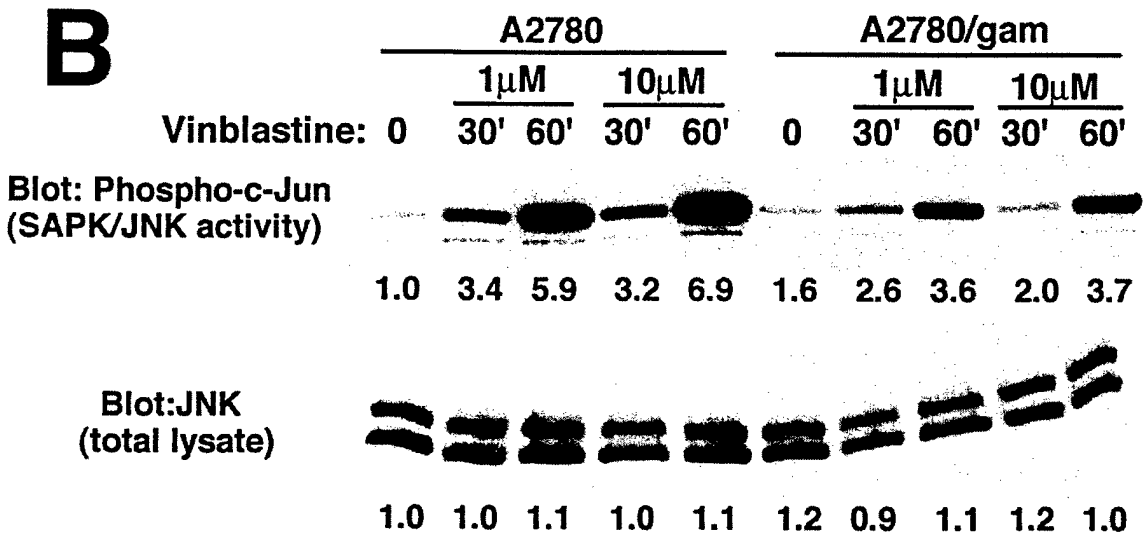


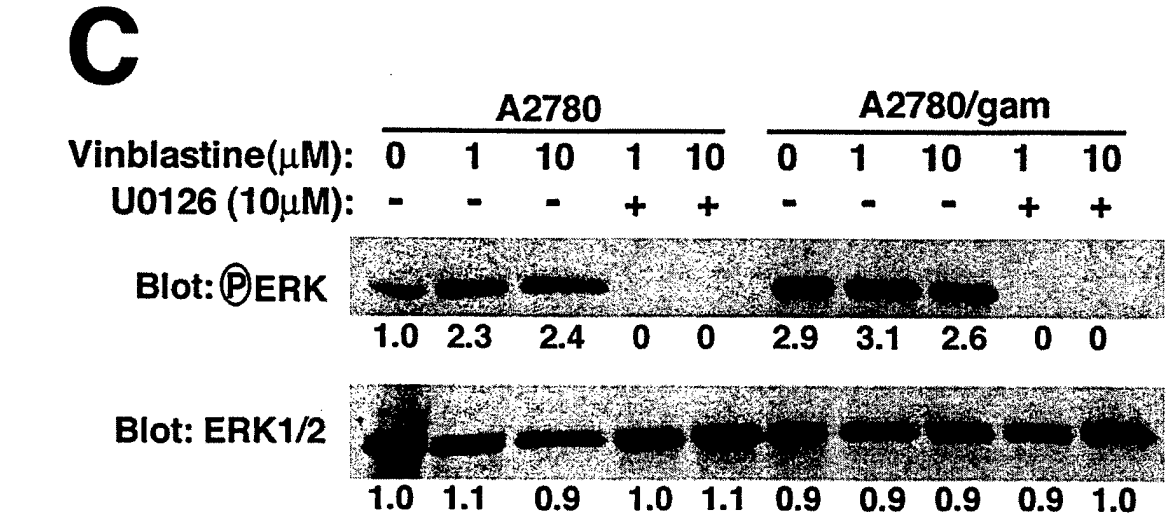
Pan et al. Fig. 8



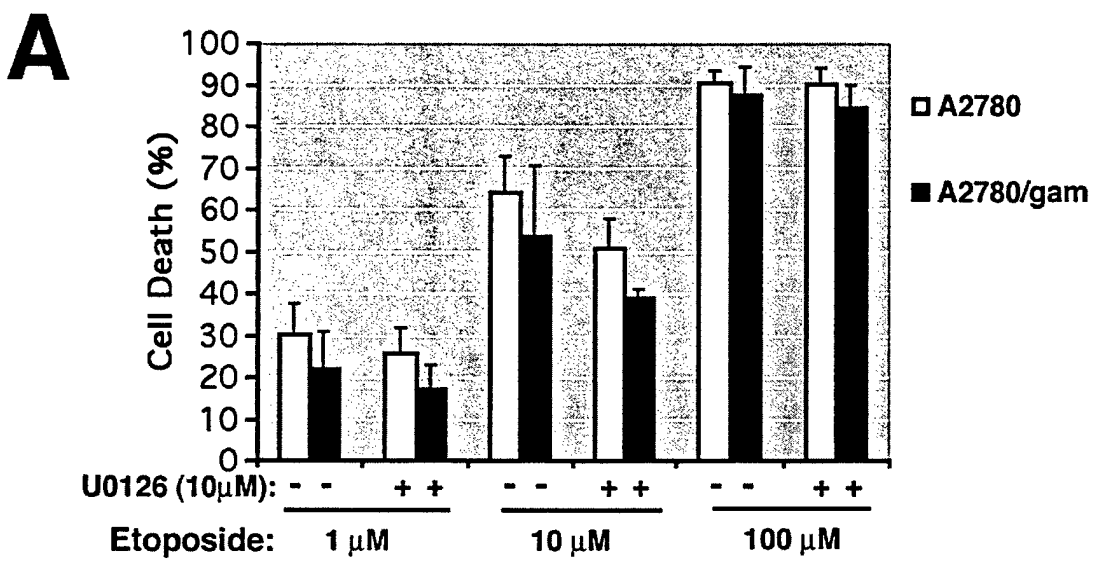
Pan et al. Fig.9

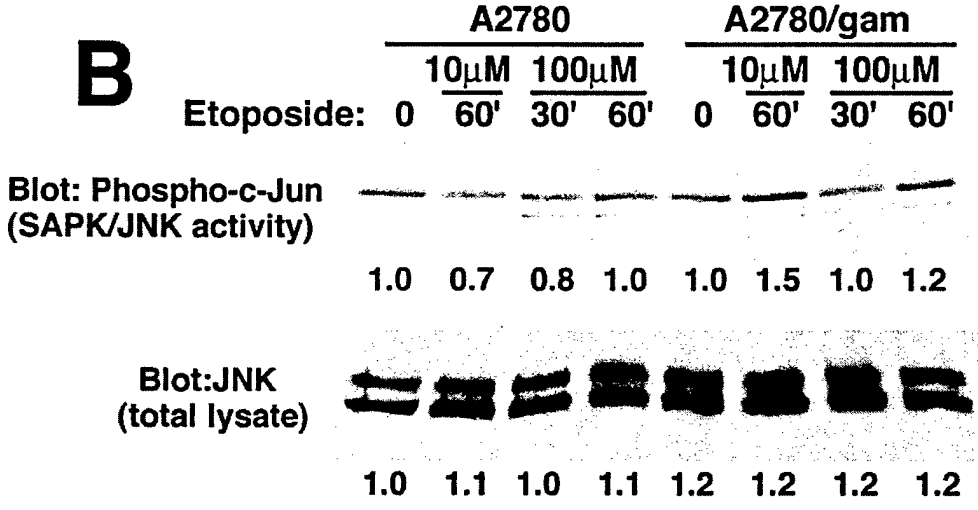


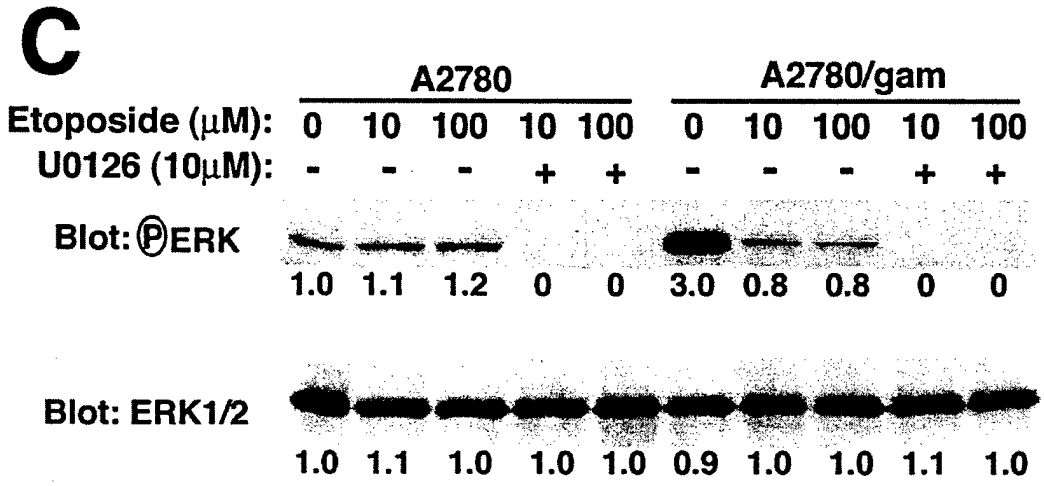




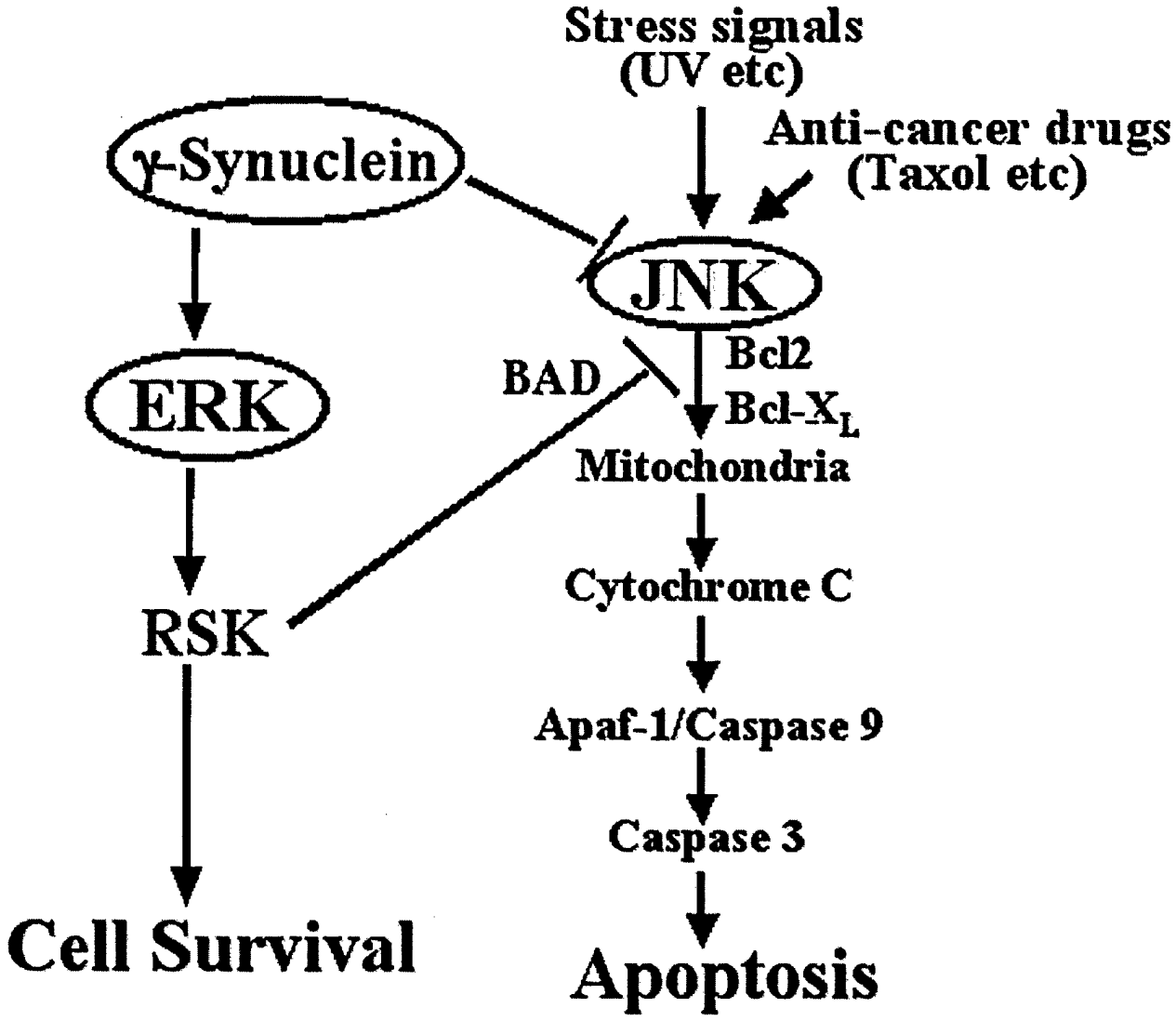
Pan et al. Fig.10







Pan et al. Fig.11



Sprouty4A, a Novel Genetic Marker of Response to Imatinib Mesylate in Gastrointestinal Stromal Tumors

Andrey Frolov[1,4], Juan Pablo Arnoletti[2], Zhong-Zong Pan[1], Jonathan Fletcher[3], Margaret von Mehren[1], Burton Eisenberg[2,6], Andrew K. Godwin[1,5,6]

[1] Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA

[2] Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, PA

[3] Dana-Farber Cancer Institute, Boston, Massachusetts

[4] Graduate Program in the Department of Molecular Biology and Biotechnology, Russian State Medical University, Moscow, Russia

Running title: *Sprouty4A* and imatinib mesylate in Gastrointestinal Stromal Tumors

Key words: *Sprouty 4A*, imatinib mesylate, gastrointestinal stromal tumors

Footnotes:

[5] To whom correspondence should be addressed at:

Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111

Tel. 215-728-2205; Fax 215-728-741

E-mail: A_Godwin@fccc.edu

[6] To whom reprint requests should be made.

The abbreviations used are: GIST, gastrointestinal stromal tumors; RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; ERK, extra-cellular signal regulated kinase; GRB2, growth factor receptor binding protein-2

Abstract

Gastrointestinal stromal tumors (GIST) are characterized by the presence of constitutively activated KIT. This observation has been successfully exploited in a clinical phase II trial with imatinib mesylate (Gleevec, Novartis), a selective tyrosine kinase inhibitor. We employed DNA microarrays to identify 14 genes that were differentially expressed between untreated and imatinib-treated human GIST cells, *in vitro*. One of these genes, *Sprouty4A* (*SPRY4A*) a regulator of tyrosine kinase-mediated signaling pathways, was dramatically down-regulated (~7-fold) for the entire duration of drug exposure (6-48h). In addition we found that imatinib inhibited KIT phosphorylation without affecting the total level of KIT protein. The constitutive activation of ERK1/2 and AKT was also inhibited without affecting the total cellular levels of either protein, however, we demonstrate that down-regulation of *SPRY4A* was dependent on the activation of the ERK1/2 pathway and was independent of AKT activation. To investigate the clinical correlation of these findings we examined GIST specimens from 6 patients on a phase II imatinib treatment trial. Tumor biopsies from three patients with non-responding GIST continued to express high levels of *SPRY4A*, whereas *SPRY4A* expression was down-regulated in GIST biopsies obtained during imatinib therapy from three patients who clinically demonstrated durable partial response. Evaluation of available tissue from one of the non-responder found that high levels of *SPRY4A* mRNA positively correlated with expression of constitutively active forms of c-KIT and ERK1/2. Furthermore, we determined that this tumor possessed a mutation

in exon 9 of c-KIT that has not previously been associated with resistance to imatinib mesylate. Overall, our studies suggest that *SPRY4A* is regulated by the c-KIT, ERK1/2 pathways and may be an important genetic marker of immediate clinical response to imatinib treatment. Our studies also emphasize the potential value of an *in vitro* cell model to assess GIST response to imatinib *in vivo,* for the purpose of identifying important genetic markers of clinical response and potential therapeutic.

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal malignancies of the GI tract. The neoplastic cells, in GISTs, appear to belong to the same lineage as the interstitial cells of Cajal (ICC), which are the pacemaker cells regulating gastrointestinal peristaltic activity[1]. GIST share many immunohistochemical, morphological, and ultrastructural features with ICCs that support this hypothesis[2]. In a small number of cases, however, these tumors can be located outside the GI tract (omentum, peritoneum, and retroperitoneum)[3] in tissues that are not known to contain ICCs, and therefore the true progenitor cell is controversial. These GI sarcomas are characterized by the presence of constitutively activated KIT (CD117), the receptor tyrosine kinase (RTK) encoded by the *c-KIT* proto-oncogene, also known as stem cell factor receptor[4,5]. c-KIT is a member of the RTK subclass III family and has structural homology to the receptors for FLT3, PDGF and macrophage colony-stimulating factor[6]. The proposed mechanism of constitutive KIT tyrosine phosphorylation in most GIST is a gain of function mutation in the c-*KIT* gene. These mutations generally involve either missense nucleotide substitutions or in-frame deletions, but a minority of GISTs has in-frame insertions, resulting in c-KIT activation. Most c-KIT mutations, in GISTs, involve exon 11, which encodes a portion of the cytoplasmic juxtamembrane domain. Smaller numbers of mutations involve exon 9 (extracellular domain), exon 13 (first part of the split tyrosine kinase domain), or exon 17 (phosphotransferase domain) [4,5,7-9].

Most GISTs are diagnosed in middle-aged to older adults and can occur anywhere in the gastrointestinal tract but predominantly are localized to the stomach[10]. Malignant GISTs often present with synchronous metastatic disease and even those amenable to complete surgical

resection often recur locally, with a 5-year actuarial survival rate of approximately 50%[11]. Patients with metastatic disease uniformly have a poor prognosis with a median survival of 6-8 months, as GISTs have been historically resistant to conventional and investigational therapy.

Imatinib (imatinib mesylate, Gleevec, Novartis, Basel, Switzerland), formerly known as STI571, is a 2-phenylaminopyrimidine derivative oral administrated drug which in pre-clinical studies inhibits c-ABL, BCR-ABL, and PDGFRA/B[12,13]. Several phase I/II clinical trials have demonstrated the efficacy of imatinib in the treatment of CML patients with the Philadelphia chromosome and the BCR/ABL translocation[14-16]. This drug has also been shown to be a specific *in vitro* inhibitor of c-KIT phosphorylation in several tumor cell lines[12,17]. After suggestive initial pre-clinical studies it was proposed that activated c-KIT was an early event in the initiation and progression of the malignant phenotype for GIST. A recent report indicated that imatinib rapidly inhibits c-KIT phosphorylation and tumor cell proliferation, while inducing apoptosis, in an established human GIST cell line (GIST882). GIST882 is an immortal GIST cell line that expresses an activating c-KIT mutation encoded by a homozygous exon 13 missense mutation (i.e., K642E amino acid substitution)[18]. The KIT RTK target has been exploited in two recently completed phase I/II clinical trials of GIST with early indications of over 50% of patients with unresectable or metastatic GISTs demonstrated a classic partial response and only 10% of patients manifested disease progression while receiving imatinib[19,20].

Despite its early clinical success there are limited pre-clinical and clinical data on the molecular targets of imatinib downstream from c-KIT. In particular there has not been a comprehensive evaluation of changes in GIST gene expression following imatinib therapy. We

hypothesized that evaluating gene expression arrays from a GIST cell line and from GIST patients pre and post-imatinib treatment could potentially identify novel genetic targets of this therapy in patients with GIST and subsequently define additional downstream mediators of response. Our strategy was to better characterize genetic markers of response to imatinib and correlate these to the GIST patient clinical status. Therefore the initial primary objectives of this study were to determine the differential *in vitro* gene expression patterns of the GIST882 cell line following imatinib treatment, to identify imatinib-specific genetic targets, and to validate those findings in clinical tumor specimens obtained from GIST patients before and during imatinib therapy.

Results:

Expression profiling of GIST cells treated with Imatinib using cDNA microarrays

To identify potential imatinib-specific genetic targets, we treated a human GIST cell line (GIST882) with 10 μM STI-571 for 0, 6, 24, or 48 hr. RNA samples labeled with Cy3- or Cy5-dyes were hybridized to 10,368-element cDNA microarrays containing known genes and ESTs. We identified a total of 14 genes, which displayed a significant change (at least 2.5-fold) in expression levels following treatment. Two genes were upregulated and 12 genes were down-regulated (**Fig. 1A**). We calculated the mean intensity ratio for all spots during the course of drug treatment and found that out of the 12 genes which showed decreased expression following drug treatment, only one transcript was dramatically down-regulated at each time point (from 4- to 13-fold) (**Fig. 1B**). Therefore, we focused our studies on this gene. Upon sequencing and database evaluation, we found that the sequence of the spotted cDNA fragment matched two isoforms of *Sprouty4*, *4A* and *4C* (**Fig. 2A**). *SPRY4A* (NCBI accession AF227516) and *SPRY4C*

(AF227517) represent alternatively splice forms of the same gene. *SPRY4A* transcript encodes for a 322 amino acid protein, while *SPRY4C* transcript encodes for a 106 amino acid polypeptide (Fig. 2A, lower panel). The *SPRY4A* transcript includes all of the *SPRY4C* sequence with extended amino and carboxy termini.

To validate the microarray results, we derived a cDNA probe that would detect both the ~5,000 base (*SPRY4A*) or the ~7,000 base (*SPRY4C*) transcripts of *Sprouty4*. Northern blot analysis indicated that *SPRY4A* was down-regulated following treatment with 10 μM imatinib (**Fig. 2B**). In comparison, *SPRY4C* transcript was not detected by Northern blotting (data not shown). We also designed a set of oligonucleotide PCR primers that would differentiate between the two *Sprouty4* transcripts (**Fig. 2A**). The oligonucleotide primer pair 1F/1R is predicted to yield a RT-PCR product of ~500 bp that would represent both *SPRY4A* and *4C*. In comparison, primer pair 2F/2R would yield a fragment of 812 bp for *SPRY4A* and a 358 bp fragment for *SPRY4C* (**Fig. 2A**). Our RT-PCR analysis confirmed that *SPRY4A*, and not 4C was constitutively expressed in GIST cells and exhibited dramatic down-regulation after drug treatment (**Fig. 2C**).

Imatinib inhibits *SPRY4A* expression through down-regulation of c-KIT, ERK1/2 and AKT.

We next examined GIST882 cells for activation of c-KIT, ERK1/2 and AKT using anti-phosphorylated antibodies. c-KIT, ERK1/2 and AKT were found to be constitutively activated in exponentially growing GIST882 cells (**Fig. 3A**). The inhibitory efficacy of the drug on the activity of c-KIT, ERK1/2 and AKT was then evaluated with different drug concentrations (1

and 10 μM) at various time intervals. We found that either drug concentration resulted in loss of phosphorylated c-KIT within 30 minutes without affecting the total level of KIT protein (**Fig. 3A**). Imatinib also potently inhibited the constitutive activation of ERK1/2 (ppERK1/2) without affecting total cellular levels of ERK1/2 (**Fig. 3A**). Inhibition of AKT took place within 30 minutes for 10 μM of imatinib and 1 hour for 1 μM without affecting the total level of AKT (**Fig. 3A**). We next evaluated Imatinib effects *SPRY4A* mRNA expression in GIST882. At both concentrations of drug, *SPRY4A* levels were noticeably decreased by 3 hrs and virtually undetectable by 6 hrs (**Fig. 3B**). Therefore, we showed in GIST882 cells that imatinib treatment resulted in decreased autophosphorylation of the mutant c-KIT polypeptide by inhibiting c-KIT kinase activity rather than by down-regulating expression of the c-KIT protein. Furthermore, this inhibition leads to down-regulation of the activation of ERK1/2 and AKT and decreased levels of *SPRY4A* mRNA.

***SPRY4A* expression is regulated by the ERK1/2 signaling pathway independent of AKT.**

Previous studies have shown that the ERK pathway positively regulates the expression of the Sprouty genes in mouse cells, and that in a limited number of tumor cell lines which exhibit constitutive activation of ERKs, *SPRY1* and/or *SPRY2* mRNA is elevated[21]. To determine if ERK1/2 pathways also regulated *SPRY4A* in tumor cells, we treated the GIST882 line with varying concentrations of U0126, a MEK inhibitor, for 6 hr and 24 hr and evaluated the expression of *SPRY4A*. We found that *SPRY4A* levels were decreased ~90% and ~70% by 6 and 24 hrs, respectively at a concentration of 30 μM U0126 (data not shown). Lower doses of U0126 had minimal effect on *SPRY4A* levels at either of the time points (data not shown). We next evaluated the levels of constitutively activated ERK1/2 following treatment with 30 μM

U0126 for 30 min, 1, 3, and 6 hrs. This treatment resulted in a complete suppression of activated ERKs by 30 min; expression of *SPRY4A* was reduced by 3 hrs and was nearly absent by 6 hrs **(Fig. 4A and 4B)**. The level of *SPRY4A* down-regulation was comparable to that seen with imatinib, except that the duration of suppression with a single dose of imatinib was more prolonged (data not shown). Interestingly, U0126 inhibits phosphorylation of AKT shortly after treatment but this effect was transient and the amount of phospho-AKT returned to the initial level within 3 hours of treatment **(Fig. 4A)**. Furthermore, the presence of U0126 did not affect the levels of total AKT. These results further confirm that constitutive activation of ERK1/2 through the c-KIT pathway leads to expression of *SPRY4A* and that this process may be independent of AKT signaling.

SPRY4A expression in clinical GIST samples.

Core biopsy tumor specimens taken from imatinib-treated GIST patients (as described in Methods) were evaluated for expression of *SPRY4A* by RT-PCR analysis. The expression levels of *SPRY4* were dramatically decreased following imatinib therapy in tumors from patients (#1,2,3) who showed a favorable clinical response to the drug **(Fig. 5A)**. In comparison, the two imatinib-resistant patients (#5 and #6) expressed similarly high levels of *SPRY4A* both pre- and post-treatment. In the patient (#4) who initially responded to the drug treatment but subsequently relapsed, the *SPRY4A* levels in the tumor decreased dramatically for the duration of the favorable clinical response but returned to the pre-treatment levels during disease progression **(Fig. 5A)**.

In the patient #6, sufficient tissue from the non-responding tumor was available to evaluate the c-KIT, AKT and ERK1/2 protein levels. A western blot of the drug resistant GIST indicated a continued expression of activated forms of c-KIT, AKT and ERK1/2 during imatinib therapy **(Fig. 6A, B, and C)**. In comparison, a control specimen taken from an untreated patient with the histological diagnosis of myxoid liposarcoma, a sarcoma histotype that is clinically unresponsive to imatinib, failed to express detectable levels of c-KIT (**Fig. 6A**). Importantly, in this control liposarcoma tissue, ERK1/2 was constitutively activated (**Fig. 6B**) and *SPRY4A* was expressed at a level comparable to a typical GIST specimen (data not shown). These results suggest that constitutive activation of ERK1/2 in GISTs and in a liposarcoma, whether c-KIT-dependent or -independent, contribute to expression of *SPRY4A*. We were also able to determine that the tumor from patient #6 possessed an in frame mutation (1530ins6) in exon 9 of c-*KIT*. Together, our results suggest that *SPRY4A* is regulated in GISTs by the c-KIT and ERK1/2 pathways and may be an important genetic marker of clinical response to imatinib treatment for these tumors. Also we propose that this exon 9 insertion of *c-KIT* may be associated with the inability of GIST cells to respond to imatinib treatment.

Discussion

The use of cDNA microarrays to identify specific genetic targets for cytotoxic agents as well as markers of drug resistance has been recently proposed[22]. A new paradigm in the clinical trial process has been described, where novel molecular targets are identified with a genomic-based approach following Phase II clinical trials ("prediction-based clinical trial process")[23]. The present study constitutes a timely effort in that direction.

We employed a genomic-based cDNA microarray approach to identify potential genetic targets of imatinib therapy in gastrointestinal stromal tumors. Our results show that ~0.1% (14 of 10,367) of the gene sequences evaluated were significally differentially expressed in a GIST cell line after inhibition of KIT oncoprotein kinase activity by imatinib. We observed that *Sprouty4A* (*SPRY4A*) mRNA levels were the most significantly down-regulated of all genes evaluated for the duration of drug treatment. With this genocentric approach we then proceeded to identify some of the upstream mediators that participated in that response. We confirmed as expected that imatinib potently inhibited the activity of c-KIT in a GIST cell line. Inhibition of KIT receptor kinase activity markedly decreased activation of the MAP kinases, ERK1 and ERK2 and the PI3K-dependent enzyme AKT. Furthermore, imatinib had no effect on total cellular expression of c-KIT, ERK1/2, or AKT. Our *in vitro* findings were then validated in a group of locally advanced or metastatic GIST patients undergoing imatinib therapy. *SPRY4A* expression was found to be a highly reliable predictor of immediate response to drug. Notably, *SPRY4A* expression was an informative response marker even in small core tumor biopsies that are limited in tissue quantity. This is important given phosphospecific antibodies often cross-react with other phosphoproteins that share related phosphorylated epitopes, and phosphospecifics are therefore, unlikely to be highly specific in paraffin section immunohistochemistry. Whereas, the prospect of developing an antibody with specificity for total Sprouty4A may be more realistic. Our study also uncovered an activating mutation in c-*KIT* that was associated with a failure to respond to imatinib. This in frame mutation (1530ins6) in exon 9 of c-*KIT* has previously been described in a study of Japanese GIST patients[24], but has not been correlated with response to imatinib. Overall, we demonstrated the potential of a GIST

cell to serve as a surrogate model to assess GIST response to imatinib *in vivo,* for the purpose of identifying important genetic markers of clinical response and potential therapeutic targets.

The cDNA gene expression array screen identified *SPRY4A* as an imatinib-responsive gene. However, little is known about the overall function of SPRY4A or whether it has a potential role in the pathogenesis of gastrointestinal stromal tumors. Sprouty was originally identified as a down-regulator of the "Breathless" (the *Drosophila* equivalent of fibroblast growth factor receptor) signaling cascade that governs tracheal branching[25]. Sprouty is an intracellular protein associated with the inner surface of the plasma membrane that binds two intracellular components of the Ras pathway, Drk (the *Drosophila* homolog of Grb2) and Gap1, a Ras GTPase-activating protein[26]. Sprouty is an inhibitor of Ras pathway signal transduction in particular and of RTK signaling in general[26,27]. While *Drosophila* has only one Sprouty protein, mammals have at least five related proteins (Sprouty 1, 2, 3, 4A, and 4C) encoded by four genes. The Sprouty proteins are classified under the same gene family by virtue of their characteristic cysteine-rich residues located in their carboxyl termini. The biological role of vertebrate Sprouty seems to be evolutionarily conserved as murine Spry2 has been shown to interfere with the FGF-mediated processes of lung morphogenesis, and murine Spry2 and Spry4 inhibit FGF-mediated bone growth and limb patterning[28-30]. The mouse Sprouty-4 protein (which has 93% homology with SPRY4A at the amino acid level) has also been shown to inhibit vascular endothelial growth factor (VEGF)-mediated angiogenesis via inhibition of RTK signaling upstream of Ras[31]. However, the precise molecular mechanisms by which Sprouty proteins negatively modulate RTK signaling pathways remain largely obscure. Sprouty proteins are thought to participate in a

negative feedback regulatory loop against signaling pathway mediators that include RTK, Ras, Raf-1 and ERKs[21].

The role of the human Sprouty proteins in cancer remains largely undefined. On the surface, loss of *SPRY4A* expression in imatinib-responsive GISTs would seem counter intuitive, given that the proposed action of Sprouty is that of a general receptor tyrosine kinase inhibitor. Release of this inhibition would seem to help promote tumorigenesis, not suppress tumor growth. For example, in mammalian cells, murine Sprouty proteins and human SPRY2 have been shown to inhibit EGF-induced cell migration and proliferation[32,33], but the biochemical basis for these effects is unknown. In contrast, Egan and colleagues have recently reported that ectopic expression of either human SPRY1 or SPRY2 did not interfere with EGF-mediated activation of MAP kinase in mammalian cells[34]. Although these observations seem to be contradictory of the genetic evidence for the function of *Drosophila* Sprouty, they are in agreement with a recent study documenting the lack of inhibitory effect of either Spry1 or Spry2 on EGF-induced MAP kinase activation in mouse endothelial cells[33]. Furthermore, recent studies have indicated that SPRY2 binds to the c-Cbl proto-oncogene product, a negative regulator of receptor and non-receptor tyrosine kinases[35]. The potentiating effects of Spry2 on EGF-induced MAP kinase activation have been attributed to its interaction with c-Cbl binding[34]. This binding to c-Cbl is N-terminal sequence dependent[35] and unlike the C-terminal regions of the Sprouty proteins, they do not exhibit a large degree of homology between family members. In this aspect, SPRY1 has also been found to bind c-Cbl, whereas SPRY4 does not[35]. Hence, one might infer that SPRY4 plays a different functional role from SPRY1 and SPRY2.

SPRY4A may also have an active role in melanocytes, mastocytes and other c-KIT expressing cells. We have also found that *SPRY4A* is constitutively expressed in liposarcomas and leiomiosarcomas (data not shown), sarcoma histotypes that as a group are unresponsive to imatinib. In this respect we have shown that c-KIT is not expressed in these tumors (**Fig. 6** and data not shown), but that ERK1/2 is constitutively activated. Therefore, it is possible that *SPRY4A* expression in these tumors may be regulated via the EGFR signaling pathway given that EGF treatment can also induce Spry4 expression in mammalian cells[36]. Sprouty has also been demonstrated to interact with Grb2[26] and Grb2, in turn, has been found to be tyrosine phosphorylated by Bcr/Abl[37]. These findings may further implicate Sprouty in the therapeutic response to imatinib seen among CML patients. The pathogenic significance of Sprouty in GISTs remains to be determined. In particular, it is unclear whether Sprouty4A plays a crucial role in propagating and/or modifying the KIT oncogenic signal, or alternately whether it might be a nonessential downstream response marker of an imatinib-induced c-KIT inhibition. During the preparation of the manuscript, the additional evidence of *SPRY4* involvement in GIST became available[38]. This report indicates that *SPRY1*, *SPRY4* and *c-KIT* are among the genes that distinguish GIST from another soft tissue sarcomas.

The MAP kinase family is composed of several sub-families including the extracellular signal-regulated protein kinases (ERK1/2), c-Jun N-terminal kinase 1 (JNK1), and p38 MAP kinase[39,40]. In the mammalian signal transduction system, GRB2 links RTK activation to the RAS family of proteins, which in turn stimulate the sequential activation of RAF serine/threonine kinases, MEK, and ERK1/2[37,41,42]. *Sprouty* gene expression is positively regulated by the ERK pathway downstream of RTK[21]. We confirmed for the first time that

SPRY4A, and not *SPRY4C* expression was dependent on activation of ERK1/2 in a clinical GIST specimen and in a GIST cell line. Furthermore, we have shown that this down-regulation is apparently independent of the hyperactivation of AKT given that treatment with a MEK inhibitor; U0126 only transiently inhibited phosphorylation of AKT while *SPRY4A* mRNA levels remained depressed (**Figs. 3 & 4**). This is potentially significant, given that the PI3K/AKT pathway is dysregulated in many human diseases and that imatinib treatment results in loss of phospho-AKT (**Fig. 3**). It will be important in future studies to determine if the therapeutic effect of imatinib is dependent on ERK1/2 and/or AKT signaling cascades. Nevertheless, we have demonstrated that expression of *SPRY4A* is a reliable marker of imatinib response in GISTs. Our findings also point to *SPRY4A* as a potential genetic endpoint of the imatinib-induced arrest of the c-KIT signaling pathway. This may translate into the clinical application of *SPRY4A* expression as a tool for early discrimination between patients that respond to therapy and those who will progress.

In summary, we have employed a genomic-based approach to identify *SPRY4A* as a genetic target of imatinib therapy in a GIST cell line and in GIST clinical specimens. We have also identified ERK1/2 signaling as a regulator of *SPRY4A* expression and as an upstream mediator of imatinib-induced therapeutic effects. Overall, we propose that the GIST cell culture model can be used effectively to assess GIST response to imatinib *in vivo,* for the purpose of identifying important genetic markers of clinical response and potential future therapeutic targets.

Materials and Methods:

GIST cell culture

GIST882 cells were maintained in F-10 media supplemented with 15% FCS, 1% Bovine Pituitary Extract (Invitrogen, Carsbad, CA), 0.5% MITO+ Serum Extender (Becton Dickinson, Bedford, MA) and L-glutamine.

GIST cells treatment with Imatinib

Imitanib mesylate (provided by Novartis Oncology) was dissolved in water at a stock concentration of 10 mM. GIST882 cells were cultured to 60-70% confluency. Forty-eight hours prior to the treatment, cells were refed with complete media. Imitanib was added directly to the media to achieve the final concentration of either 1 μM or 10 μM.

Total RNA preparation

Imatinib treated and control GIST882 cells were harvested simultaneously at 60-80% confluency. Total RNA was isolated using guanidinium/isothiocyanate/phenol/chloroform method as previously described[43]. Total RNA was separated by agarose gel electrophoresis and visualized by ethidium bromide staining to check for integrity. 100 μg of total RNA was DNase treated using "DNA free" kit (Ambion, Austin, TX) according to manufacturer's specifications. Following DNase treatment the RNA was quantified and evaluated for integrity by agarose electrophoresis.

Microarray preparation

10,367 human cDNA fragments corresponding to known genes and ESTs from the I.M.A.G.E. consortium library (Research Genetics) were amplified by polymerase chain reaction

(PCR), purified by isopropanol precipitation and resuspended in 50% DMSO at a concentration of 150 ng/μl. Arrays were spotted on GeneMachine Omnigrid arrayer (GeneMachine, San Carlos, CA) using poly-Lysine coated glass slides. Slides were baked for 3 hours at 80°C in a vacuum oven, crosslinked in UV light (90 mJ) in Stratalinker (Stratagene, La Jolla, CA) and processed as described in MGuide at http://smgm.stanford.edu/pbrown/mguide/index.html.

Preparation and hybridization of the probe cDNA.

Fifteen micrograms of total RNA were reverse transcribed and amino allyl dUTP was incorporated in a reaction containing 500ng oligo (dT) primers, 1x first strand buffer (Invitrogen, Carlsbad, CA), 0.01M DTT, 500 μM each of dATP, dCTP, dGTP, and dTTP/aadUTP (2:3 ratio) 40Units of rRNasin (Promega, Madison, WI) and 200Units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). After brief denaturation and annealing of the primers at 70°C for 8 mins, the reaction was incubated at 42°C for 2 hours, followed by alkali hydrolysis of RNA and cDNA purification using Microcon-30 columns (Millipore, Bedford, MA) according to the manufacturer's instructions. The cDNA was then labeled with either Cy3 or Cy5 dyes by a coupling reaction using FluoroLink ™ Cy3 and Cy5 monofunctional dyes (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacture's specifications. Probes were the purified using StrataPrep PCR Purification Kit (Stratagene, La Jolla, CA). Two of the samples (one labeled with Cy3 and one with Cy5) were combined, denatured, pre-annealed in the presence of 10 μg of Cot-1 DNA (Invitrogen, Carlsbad, CA) and 10 μg of poly-dA DNA, and hybridized to the cDNA microarrays overnight at 42°C in hybridization buffer (25% formamide, 5x SSC, 0.1% SDS and 100 μg/ml sonicated salmon sperm DNA). After hybridization, the arrays were washed 2 times in washing buffer A (2 x SSC, 0.1% SDS) at 42°C for 5 minutes and

then 3 times in washing buffer B (0.1 x SSC, 0.1% SDS) at room temperature for 10 minutes. The slides were briefly dipped into distilled water and dried in a stream of nitrogen before scanning.

Intensity extraction and data analysis.

Images were obtained by scanning the arrays in Affymetrix 428 scanners. Signal intensities for Cy3- and Cy5-labeled probes were extracted by ImaGene software package version 4.2 (BioDiscovery, Inc., Marina Del Rey, CA) using default settings and auto segmentation. Mean intensities for signal and background as well quality characteristics ("empty" or "poor") of the spots were obtained at this time. The threshold for empty spots was achieved by raising threshold to a point when all blank spots were flagged. The formula for determining this value is as follows: if $\frac{(M_S - M_B)}{\sigma_B}$ < threshold, then the spot is flagged, where M_S is the mean of signal, M_B is the mean of background and σ_B is the Standard Deviation of the background. The "poor" spots were calculated using the following formula if $\frac{\sigma_S}{M_S}$ > threshold, then the spot is flagged (σ_S is the Standard Deviation of the signal and M_S is the Mean of signal). The threshold was set at 0.4 to determine "poor" spots. The data were then analyzed using GeneSight software package version 3.0.4 (BioDiscovery, Inc., Marina Del Rey, CA). Data preparation consisted of the following steps: 1) background correction was performed by subtracting the local group median (clique size = 5) background from the signal of each spot; 2) spots that were flagged as "empty" and "poor" were omitted from the analysis; 3) the data were normalized using Piece-wise linear normalization, which was done using 10 bins and a minimum of 100 elements in each bin; 4) the intensity ratio of two channels was calculated; 5) the $\log_2$ was

calculated for each ratio; and 6) the two channels were normalized by subtracting the $\log_2$ of the mean of the intensity of the total signal from each individual spot intensity. At least two "flip-dye" experiments were performed for each time point and the mean of the repeated experiments were used in the final data analysis. Finally, the mean ratio for each spot was calculated for all time points and the standard deviation and coefficient of variance were calculated for these values.

cDNA microarray clone verification and sequence analysis

cDNA fragments of interested were obtained from original stock plates used for microarray fabrication by either plasmid isolation or direct amplification of the fragments from the bacteria. Clones were re-sequenced and the correct annotation and homology was identified using the basic local alignment search tool (BLAST, NCBI) against GenBank/EMBL database. DNA and amino acid sequence analyses were performed with the Wisconsin Package version 9.1 (Genetics Computer Group).

Northern Blotting

Fifteen micrograms of total RNA was used for standard Northern blot analysis, as previously described[43]. Blots were hybridized with a 1,454 bp ^{32}P-labeled *SPRY4A* cDNA probe, corresponding to nucleotides 2594-4048 of GenBank entry AF227516.

Reverse transcription of RNA

Two micrograms of DNase treated RNA were reverse transcribed using M-MuLV reverse transcriptase (NEB, Beverly, MA) in a 20 μl reaction containing 1 x RT Buffer (NEB, Beverly,

MA), 0.5 mM each dNTPs, 4 μM oligo-dT (16-18) primer, 10 units RNase inhibitor (Promega, Madison, WI) and 200 units of reverse transcriptase. Primers were pre-annealed for 10 min at 70°C, and the reaction incubated for 1 hr at 42°C followed by enzyme inactivation for 10 min at 90°C.

Polymerase chain reaction

DNA fragments of the, *SPRY4A*/C, *SPRY4A* and *β-actin* were amplified by polymerase chain reaction (PCR) using cDNAs obtained by reverse transcription of mRNA from GIST cells and tumor biopsies as a template and the following sets of primers: *SPRY4A* - forward 5'-CCGTTCTGTGGAGAGTCGATTTAC-3', reverse - 5'-GTCCCTCAGTGGCTCTCGACT-3'; Sprouty4a/4c (isoform determining) – forward - 5'-ACCATCCTACCCATTGACCA-3', reverse 5'-GGCTTCGACACAAACTGTCA-3'; β-actin – forward – 5'-CTCACCATGGATGATGATATCGC-3', reverse – 5'-CATGATGGAGTTGAAGGTAGTTTCGT-3'. PCR was performed in a reaction volume of 30 μl containing cDNA from 1 μl of the reverse transcription reaction described above as a template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 μM of both the forward and reverse primer, 60 μM of each deoxyribonucleotide, 5% dimethyl sulfoxide, and 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Following an initial denaturation step at 95°C for 5 min, DNA was amplified through 21, 23, 25, and 27 cycles consisting of 5 sec denaturing at 95°C, 60 sec annealing at 55°C, and 90 sec extension at 72°C. The products were resolved on a 1.5% agarose gel and were visualized by UV light following staining with Ethidium Bromide. Images of the gels were obtained using Alpha Imager 2200 documentation and analysis system (Alpha Innotech, San Leandro, CA). Images were captured

within linear dynamic range and controlled for white color saturation. DNA bands were quantified using Alpha Imager v5.5 software package and Fuji Image Gauge v3.11 software package (Fuji Photo Film Co., Ltd).

GIST cells treatments with MEK inhibitor

The MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI) and dissolved in DMSO at a stock concentration of 15 mM. GIST cells were cultured to 60-70% confluency. Forty-eight hours prior the treatment, the media was replaced. MEK inhibitor was added directly to the media to achieve the final concentration of 1 μM, 10 μM and 30 μM.

Cell lysate preparation, SDS-PAGE and Western blot analysis

Anti-β-actin was purchased from Sigma (St. Louis, MO) and was probed in 1:5000 dilution in 5% dried milk. Anti-phospho-c-Kit (Tyr719) was purchased from Cell Signaling (Beverly, MA) and was probed in 1:500 dilution in 5%BSA. Anti-c-KIT was purchased from Santa Cruz (Santa Cruz, CA) and was used in 1:100 dilution in 5% dried milk. Anti-phospho-ERK1/2 was purchased from Cell Signaling (Beverly, MA), anti-ERK1, and anti-ERK2 were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), anti-AKT and anti-AKT/phosphoThr^{308} antibodies were obtained from Cell Signaling, Technology (Beverly, MA) and were probed in 1:1000 dilution in 5% BSA.

Cells at 60-70% confluence were washed twice with ice-cold D-PBS before scraping on ice with Lysis Buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM Na-pyrophosphate; 1 mM Na-β-glycerophosphate; 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100, and 1

tablet of protease inhibitor cocktail (Roche, Indianapolis, IN) per 40 ml lysis buffer]. Cellular debris was removed by centrifugation (14,000 x g for 15 min at 4°C), and quantitation of protein was performed using a bicinchoninic acid/copper (II) sulfate assay (Sigma, St. Louis, MO). Fifty micrograms of total protein extract cell extracts and tissues were separated by standard SDS-PAGE and transferred to Immobilon-P (polyvinylidene difluoride; Millipore, Bedford, MA). Prior to antibody probing, the membranes were blocked in 5% dried milk. The primary antibodies were diluted 1:1000 (unless other specified), and the HRP-conjugated second antibodies were diluted 1:10,000 (Amersham, Piscataway, NJ). NEN Renaissance Enhanced Luminol Reagents (Boston, MA) were used as substrates for detection. For reuse of the same membrane with another primary antibody, the membrane with stripped with Restore Western Blot Stripping buffer (Pierce, Rocklord, IL).

Clinical samples

Tumor specimens were obtained from six patients enrolled in the CSTI571-B2222 clinical trial sponsored by Novartis Oncology. Eligibility criteria included histological confirmation of GIST with documentation of c-KIT expression, as well as evidence of unresectable recurrent and/or metastatic disease. After signing informed consent, patients were evaluated to confirm acceptable hematologic, renal and hepatic function. Patients were then randomized to receive 400 mg or 600 mg PO once daily dosing of imatinib. Ultrasound-guided 14 gauge core biopsies of non-necrotic tumor were obtained prior to therapy initiation and while on drug for between 1-28 days. Specimens were flash frozen and kept in liquid N_2. Patients were monitored for response to imatinib mesylate at 4-12 week intervals by one of the authors (M.vM.).

Patients #1-3 had GIST of either the stomach or the small bowel and had a partial response to imatinib treatment defined as greater than or equal to 50% decrease from baseline in the sum of products of perpendicular diameters of all measurable lesions. Patient #4 had a partial response to imatinib initially but developed disease progression 3 months after initiation of the treatment. The patient was taken off drug and a core tumor biopsy was obtained. Patients #5 and #6 presented with large intestine and gastric GIST respectively and they had no response to imatinib treatment, one of them (#5) remaining with stable disease and the other one developing disease progression (#6). Additional tumor was obtained from patient #6 during palliative surgical resection approximately one week after discontinuing drug therapy.

Total RNA from the tumor specimens was isolated using Trizol reagent (Invitrogen, Carlbad, CA) using manufacturer's protocol. Protein extracts from a patient with a myxoid extremity liposarcoma and GIST patient #6 tumor were prepared as described above.

Acknowledgements:

We would like to acknowledge Dr. Alfred Knudson for his insightful comments regarding the manuscript, Dr. Michael Ochs and the Bioinformatics Facility of FCCC for advice and consultation on DNA microarray data analysis, Sharon Howard and the Tissue Culture Facility for technical assistance, and the DNA array facility, the DNA sequencing facility, and the Biosample Repository/Protocol Support Laboratories at Fox Chase for services and specimens. We would also like to thank Dr. Rosaleen Parsons and Dr. Barton Milestone for their expert assistance obtaining the core biopsies and Monica Davey, RN, for her excellent care of the study patients and data management. Z.Z.P. was supported by a fellowship from the Department of

Defense (Institutional Breast Cancer Training Program grant DAMD17-00-1-0249). This work was supported in part by the Eileen-Stein Jacoby Fund, the Pardee foundation and by an appropriation from the Commonwealth of Pennsylvania.

Figure Legends:

Figure 1. cDNA microarray analysis of imatinib treated GIST882. A) Genes that were differentially expressed upon treatment with imatinib. Shown are the $\log_2$ intensity ratios for each time point and the overall mean $\log_2$ ratio for each gene. StDev=Standard deviation, CV=Coefficient of variance, EST=Expressed sequence tag. B) cDNA microarray analysis of *SPRY4A* expression. Snapshots of actual hybridization signal pairs from spotted *SPRY4A* cDNA fragment when hybridized with Cy3- and Cy5- labeled cDNA from GIST cells treated and untreated with 10 μM imatinib for 6, 12, 24, and 48 hours. Last pair (C) represents hybridization signals of β-actin in both channels as a control.

Figure 2. Structure of *SPRY4* genes and validation of the microarray experiments. **A)** Schematic and amino acid alignment of *SPRY4A* and *SPRY4C*. Diagonally shaded areas represent sequence unique for *SPRY4A*, vertically shaded area is the sequence unique for *SPRY4C*, black box represents the fragment of the cDNA sequence spotted on the microarray, arrows indicate the position of primer sets used for RT-PCR analysis. In the lower panel, the amino acid sequence comparison for SPRY4A and SPRY4C are shown. Shaded regions indicated regions of sequence identity. **B)** Northern blot analysis of *SPRY4A* expression. Upper panel: the *SPRY4A* mRNA levels in imatinib treated and control GIST cells. Lower panel: Ethidium bromide-stained gel prior to blotting. The position of the 28s and 18s rRNA is

indicated. **C)** RT-PCR analysis of *SPRY4* expression. Upper panel: Isoform determination and the level of the *SPRY4* RT-PCR product in imatinib treated and untreated GIST cells. Lower panel: β-actin RT-PCR product levels in drug treated and untreated GIST cells.

Figure 3. Imatinib treatment response in GIST cells. **A)** Analysis of phospho-c-Kit/total c-Kit, phospho-AKT/total AKT, phospho-ERK1/2/total ERK1/2 levels in drug treated and untreated GIST cells by immunoblotting. **B)** RT-PCR analysis of *SPRY4A* and β-actin levels in imatinib treated and untreated GIST cells.

Figure 4. Treatment of GIST cells with a MEK inhibitor (U0126) leads to decreased levels of activated ERK1/2 levels and to reduced levels of *SPRY4A*. **A)** Analysis of phospho-ERK1/2/total ERK1/2, phospho-AKT/total AKT levels in U0126 treated and untreated GIST cells by immunoblotting. **B)** RT-PCR analysis of *SPRY4A* and β-actin levels in U0126 treated and untreated GIST cells.

Figure 5. Analysis of *SPRY4A* expression in response to imatinib treatment in clinical cases of GIST. Upper panel: Levels of *SPRY4A* RT-PCR product before and after drug administration in six patients with GIST [patients #1, 2, 3 responded favorably to the drug, patient #4 initially responded but subsequently progression (R-P), and patients #5 and 6 failed to respond to treatment (NR)]. Lower panel: β-actin RT-PCR product levels as a control.

Figure 6. c-KIT, ERK1/2 and AKT expression in liposarcoma, GIST cells and imatinib resistant GIST by immunoblotting. **A)** Phospho-c-KIT and total c-KIT protein; **B)** phospho-AKT and total

AKT levels; and C) phospho-ERK1/2 and total ERK1/2 levels in tumor and tumor cell line extracts.

References:

1. Graadt van Roggen, J.F., van Velthuysen, M.L. & Hogendoorn, P.C. The histopathological differential diagnosis of gastrointestinal stromal tumours. *J Clin Pathol* **54**, 96-102. (2001).
2. Rumessen, J.J., Peters, S. & Thuneberg, L. Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. *Lab. Invest.* **68**, 481-495 (1993).
3. Miettinen, M. et al. Gastrointestinal stromal tumors/smooth muscle tumors (GISTs) primary in the omentum and mesentery: clinicopathologic and immunohistochemical study of 26 cases. *Am. J. Surg. Pathol.* **23**, 1109-1118 (1999).
4. Lasota, J., Jasinski, M., Sarlomo-Rikala, M. & Miettinen, M. Mutations in exon 11 of c-Kit occur preferentially in malignant *versus* benign gastrointestinal stromal tumors and do not occur in leiomyomas or leiomyosarcomas. *Am. J. Pathol.* **154**, 53-60 (1999).
5. Rubin, B.P. et al. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. *Cancer Res.* **61**, 8118-8121 (2001).
6. Qiu, F.H. et al. Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family--oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *Embo J* **7**, 1003-11. (1988).
7. Hirota, S. et al. Gain-of-function mutations of c-*kit* in human gastrointestinal stromal tumors. *Science* **279**, 577-580 (1998).

8. Nakahara, M. et al. A novel gain-of-function mutation of c-*kit* gene in gastrointestinal stromal tumors. *Gastroenterology* **115**, 1090-1095 (1998).

9. Lux, M.L. et al. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. *Am. J. Pathol.* **156**, 791-795 (2000).

10. Miettinen, M., Sarlomo-Rikala, M. & Lasota, J. Gastrointestinal stromal tumors: recent advances in understanding of their biology. *Hum. Pathol.* **30**, 1213-1220 (1999).

11. DeMatteo, R.P. et al. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. *Ann. Surg.* **231**, 51-58 (2000).

12. Buchdunger, E. et al. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J. Pharmacol. Exp. Ther.* **295**, 139-145 (2000).

13. Druker, B.J. STI571 (Gleevec) as a paradigm for cancer therapy. *Trends Mol Med* **8**, S14-8. (2002).

14. Kantarjian, H. et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N. Engl. J. Med.* **346**, 645-652 (2002).

15. Talpaz, M., Silver, R.T., Druker, B. & al., e. Gleevec (formerly ST1571): an active drug in patients with Ph+ chronic myeloid leukemia in accelerated phase - updated results of a phase II study. *Blood* **98**, 845a (2001).

16. Sawyers, C.L. et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* **99**, 3530-9. (2002).

17. Heinrich, M.C. et al. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood* **96**, 925-932 (2000).

18. Tuveson, D.A. et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene* **20**, 5054-5058 (2001).

19. Blanke, C.D., von Mehren, M., Joensuu, H. & al., e. High incidence of durable responses induced by imatinib mesylate (Gleevec) in patients with unresectable and metastatic gastrointestinal stromal tumors (GISTs). *Prog. Proc. Am. Soc. Clin. Oncol.* **21**, 1608a (2002).

20. van Oosterom, A.T. et al. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *Lancet* **358**, 1421-1423 (2001).

21. Ozaki, K. et al. ERK pathway positively regulates the expression of Sprouty genes. *Biochem Biophys Res Commun* **285**, 1084-8. (2001).

22. Rosell, R. et al. Determinants of response and resistance to cytotoxics. *Semin. Oncol.* **29**, 110-118 (2002).

23. Paik, S. Incorporating genomics into the cancer clinical trial process. *Semin Oncol* **28**, 305-9. (2001).

24. Sakurai, S. et al. Mutations in c-kit gene exons 9 and 13 in gastrointestinal stromal tumors among Japanese. *Jpn J Cancer Res* **92**, 494-8. (2001).

25. Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. & Krasnow, M.A. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **92**, 253-63. (1998).

26. Casci, T., Vinos, J. & Freeman, M. Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-65. (1999).

27. Reich, A., Sapir, A. & Shilo, B. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* **126**, 4139-47. (1999).

28. Minowada, G. et al. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-75. (1999).

29. Tefft, J.D. et al. Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. *Curr Biol* **9**, 219-22. (1999).

30. Mailleux, A.A. et al. Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech Dev* **102**, 81-94. (2001).

31. Lee, S.H., Schloss, D.J., Jarvis, L., Krasnow, M.A. & Swain, J.L. Inhibition of angiogenesis by a mouse sprouty protein. *J Biol Chem* **276**, 4128-33. (2001).

32. Yigzaw, Y., Cartin, L., Pierre, S., Scholich, K. & Patel, T.B. The C terminus of sprouty is important for modulation of cellular migration and proliferation. *J Biol Chem* **276**, 22742-7. (2001).

33. Impagnatiello, M.A. et al. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *J Cell Biol* **152**, 1087-98. (2001).

34. Egan, J.E., Hall, A.B., Yatsula, B.A. & Bar-Sagi, D. The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc Natl Acad Sci U S A* **99**, 6041-6. (2002).

35. Wong, E.S., Lim, J., Low, B.C., Chen, Q. & Guy, G.R. Evidence for direct interaction between Sprouty and Cbl. *J Biol Chem* **276**, 5866-75. (2001).

36. Sasaki, A., Taketomi, T., Wakioka, T., Kato, R. & Yoshimura, A. Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor- but not epidermal growth factor- induced ERK activation. *J Biol Chem* **276**, 36804-8. (2001).

37. Li, S., Couvillon, A.D., Brasher, B.B. & Van Etten, R.A. Tyrosine phosphorylation of Grb2 by Bcr/Abl and epidermal growth factor receptor: a novel regulatory mechanism for tyrosine kinase signaling. *Embo J* **20**, 6793-804. (2001).

38. Nielsen, T.O. et al. Molecular characterisation of soft tissue tumours: a gene expression study. *Lancet* **359**, 1301-7. (2002).

39. Ferrell, J.E., Jr. Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem Sci* **21**, 460-6. (1996).

40. Sturgill, T.W. & Wu, J. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim Biophys Acta* **1092**, 350-7. (1991).

41. Marais, R. & Marshall, C.J. Control of the ERK MAP kinase cascade by Ras and Raf. *Cancer Surv* **27**, 101-25 (1996).

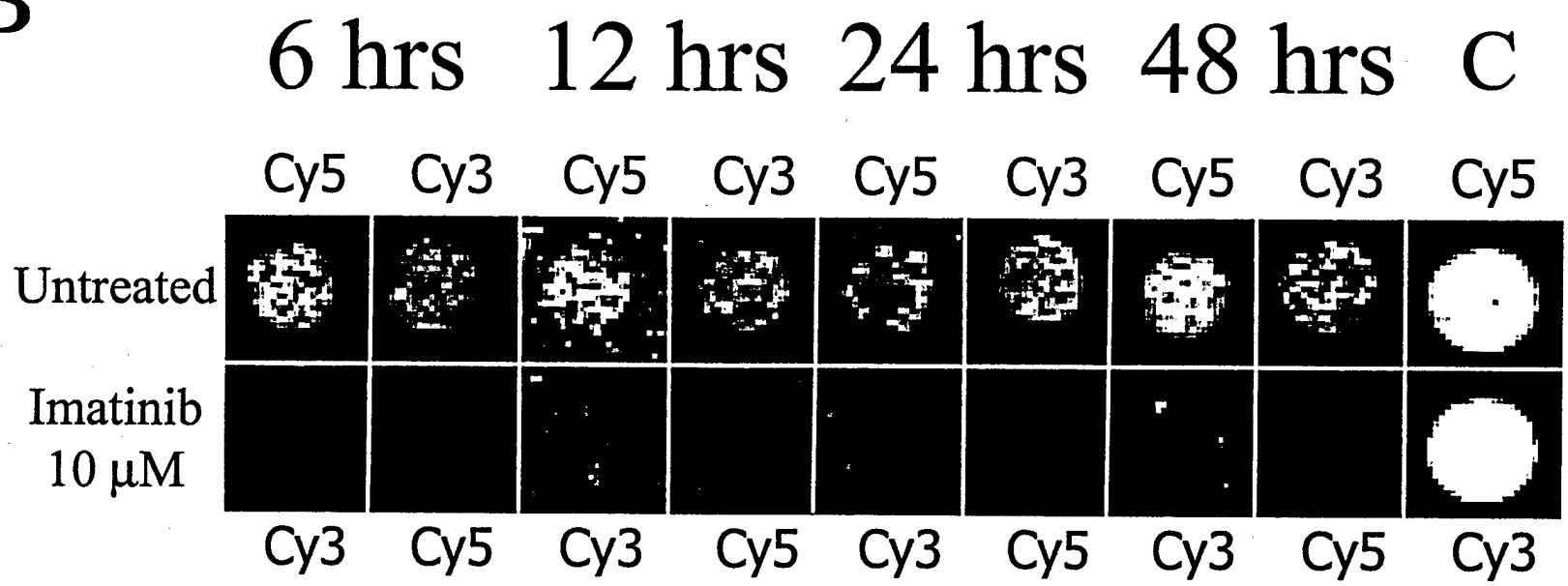
42. Segal, R.A. & Greenberg, M.E. Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci* **19**, 463-89 (1996).

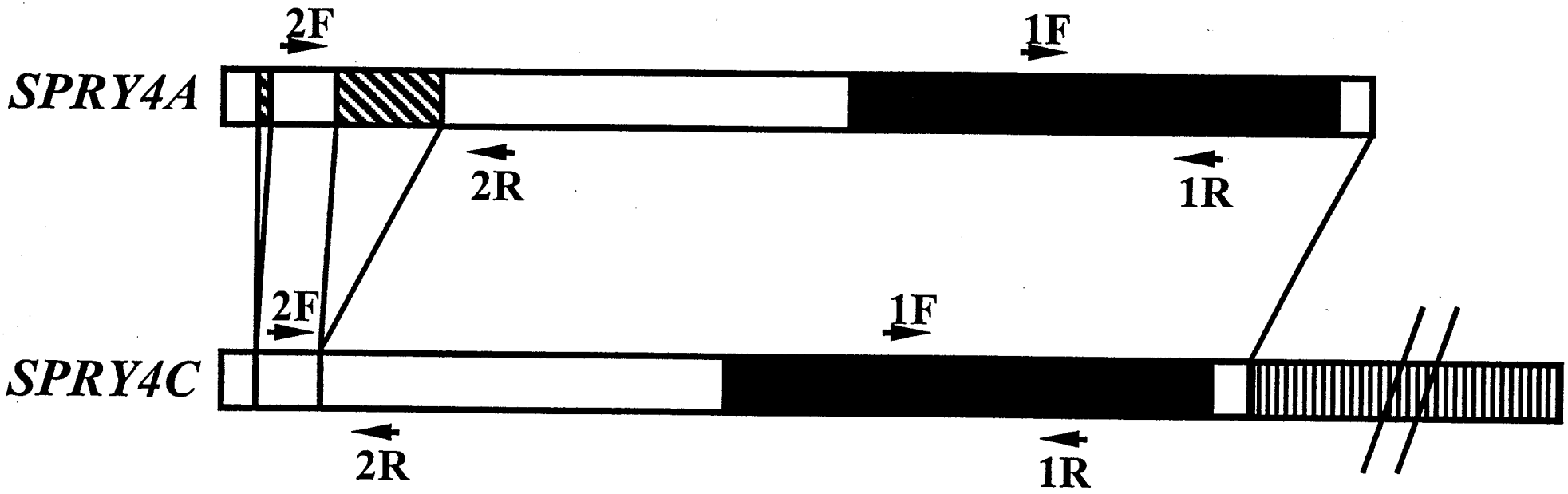
43. Schultz, D.C. et al. Identification of two candidate tumor suppressor genes on chromosome 17p13.3. *Cancer Res* **56**, 1997-2002. (1996).

A

Clone ID	6 hours	12 hours	24 hours	48 hours	Mean ratio	CV
Sprouty (Drosophila) homolog 4	-3.31	-1.91	-3.73	-2.38	**-2.83 ± 0.83**	**0.29**
EST	-2.22	-1.39	-2.54	-0.97	**-1.78 ± 0.72**	**0.41**
Hypothetical protein RTP801	-2.11	-1.17	-1.67	-1.35	**-1.58 ± 0.41**	**0.26**
EST	-1.89	-0.67	-2.29	-1.46	**-1.58 ± 0.69**	**0.44**
Hypothetical protein FLJ20898	-1.63	-0.45	-2.50	-1.49	**-1.52 ± 0.84**	**0.55**
EST (chromosome15)	-0.93	-1.43	-1.72	-1.70	**-1.45 ± 0.37**	**0.26**
Homo sapiens, clone MGC:3182, mRNA	-1.95	-0.80	-1.39	-1.49	**-1.41 ± 0.48**	**0.34**
EST	-0.86	-1.03	-2.42	-0.99	**-1.32 ± 0.73**	**0.55**
EST	-0.87	-0.81	-1.53	-1.99	**-1.30 ± 0.57**	**0.44**
Solute carrier family 1	-0.34	-0.88	-2.93	-1.01	**-1.29 ± 1.13**	**0.88**
Phosphodiesterase 2A, cGMP-stimulated	-0.45	-0.72	-2.63	-1.25	**-1.26 ± 0.97**	**0.77**
EST	1.81	1.13	1.61	0.72	**1.32 ± 0.49**	**0.37**
EST	0.92	2.16	1.93	0.49	**1.38 ± 0.80**	**0.58**

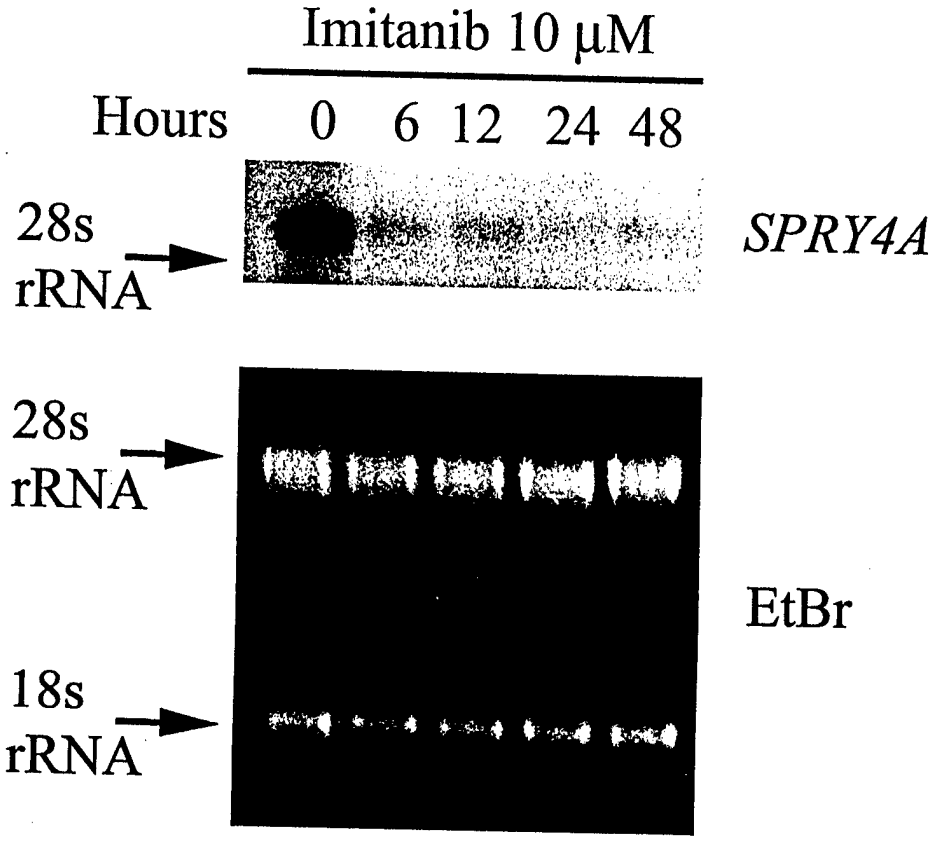
B

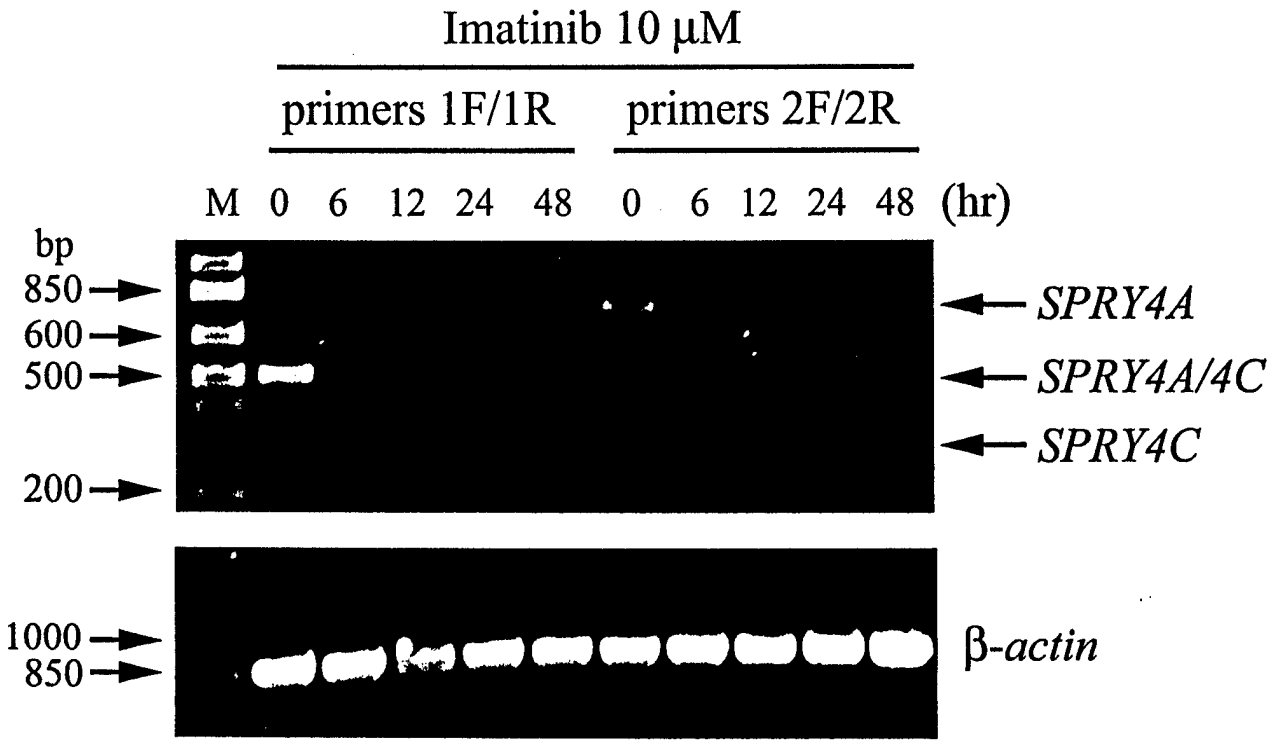


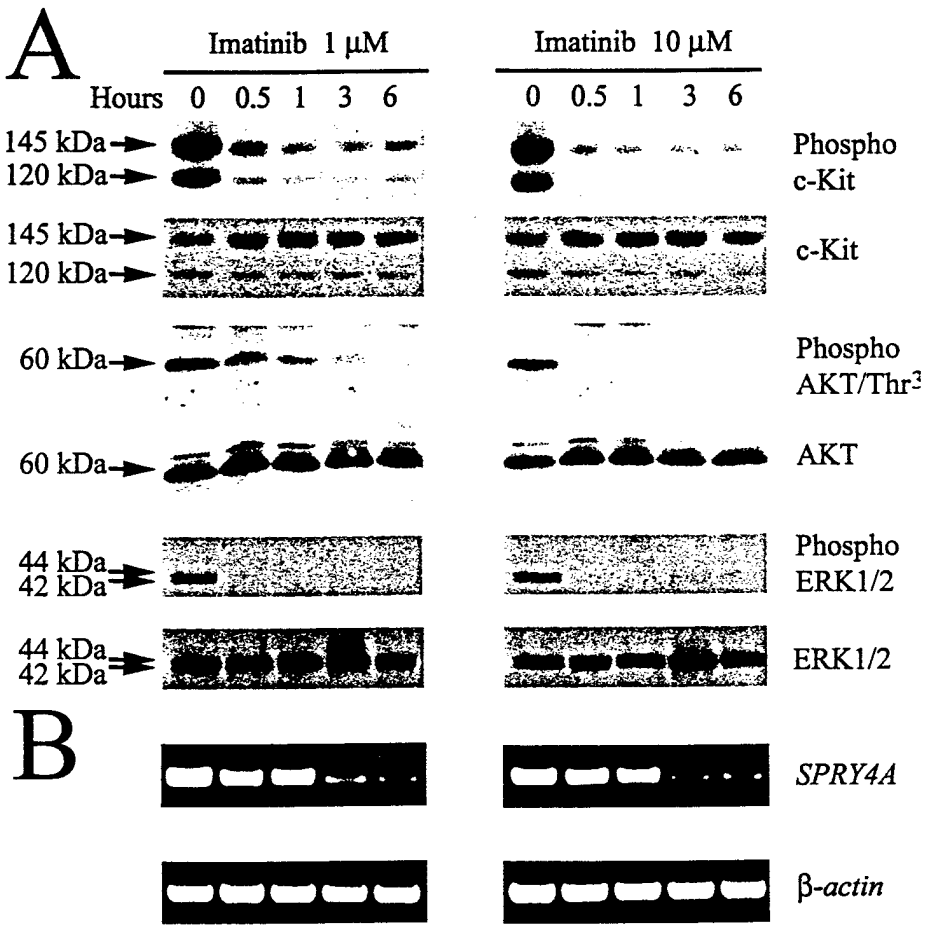


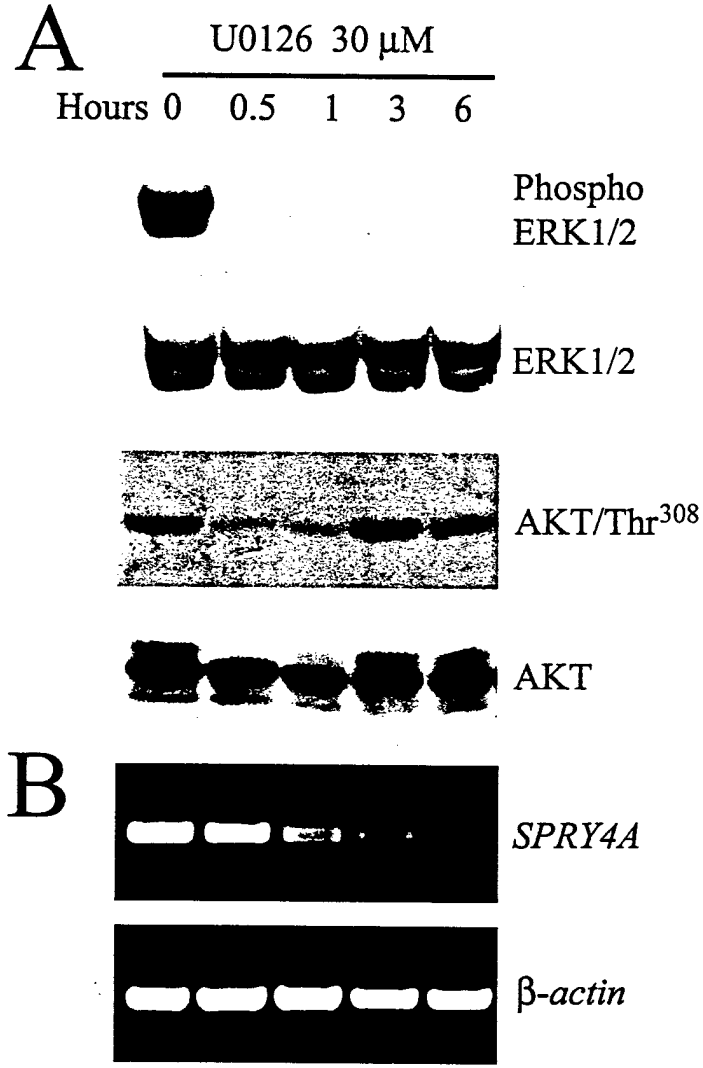
500 bp

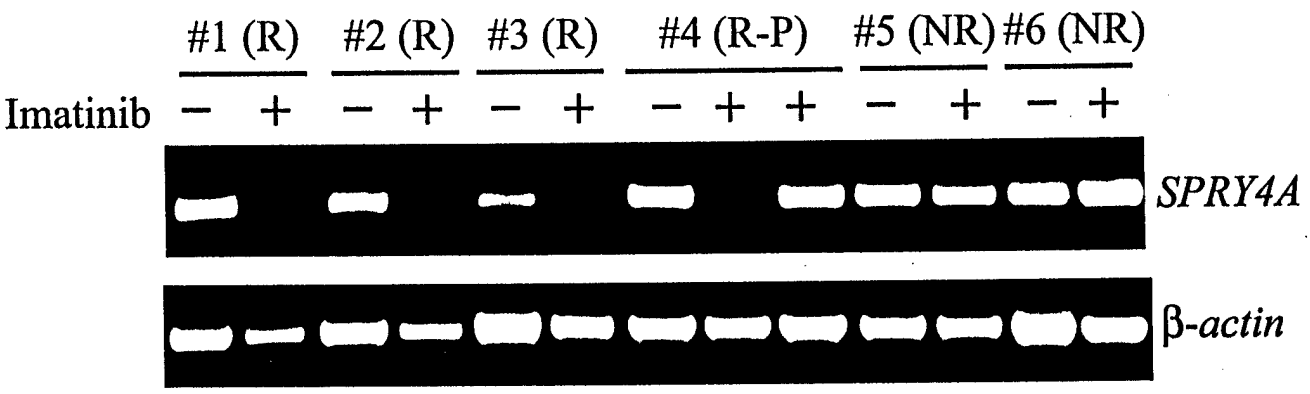
```
                1          11         21         31         41         51         61         71         81         91
SPRY4c          ----------------------MEPPIPQSAPLTPNSVMVQPLLDSRMSHSRLQHPLTILPIDQVKTSHVENDYIDNPSLALTTGPKRTRGGAPELAPT
SPRY4a          MLSPLPTGPLEACFSVQSRTSSPMEPPIPQSAPLTPNSVMVQPLLDSRMSHSRLQHPLTILPIDQVKTSHVENDYIDNPSLALTTGPKRTRGGAPELAPT
Consensus                              meppipqsapltpnsvmvqplldsrmshsrlqhpltilpidqvktshvendyidnpslalttgpkrtrggapelapt

                101        111        121        131        141        151        161        171        181        191
SPRY4c          PARCDQDVTHHWISFSGRPCSAT----------------CLPPAA----------------------------------------------------------
SPRY4a          PARCDQDVTHHWISFSGRPSSVSSSSSTSSDQRLLDHMAPPPVADQASPRAVRIQPKVVHCQPLDLKGPAVPPELDKHFLLCEACGKCKCKECASPRTLP
Consensus       parcdqdvthhwisfsgrp s                  pp a

                201        211        221        231        241        251        261        271        281        291
SPRY4c          ----------------------------------------------------------------------------------------------------
SPRY4a          SCWVCNQECLCSAQTLVNYGTCMCLVQGIFYHCTNEDDEGSCADHPCSCSRSNCCARWSFMGALSVVLPCLLCYLPATGCVKLAQRGYDRLRRPGCRCKH
Consensus

                301        311        321        331        341        351        361        371        381        391
SPRY4c          ----------------------
SPRY4a          TNSVICKAASGDAKTSRPDKPF
Consensus
```

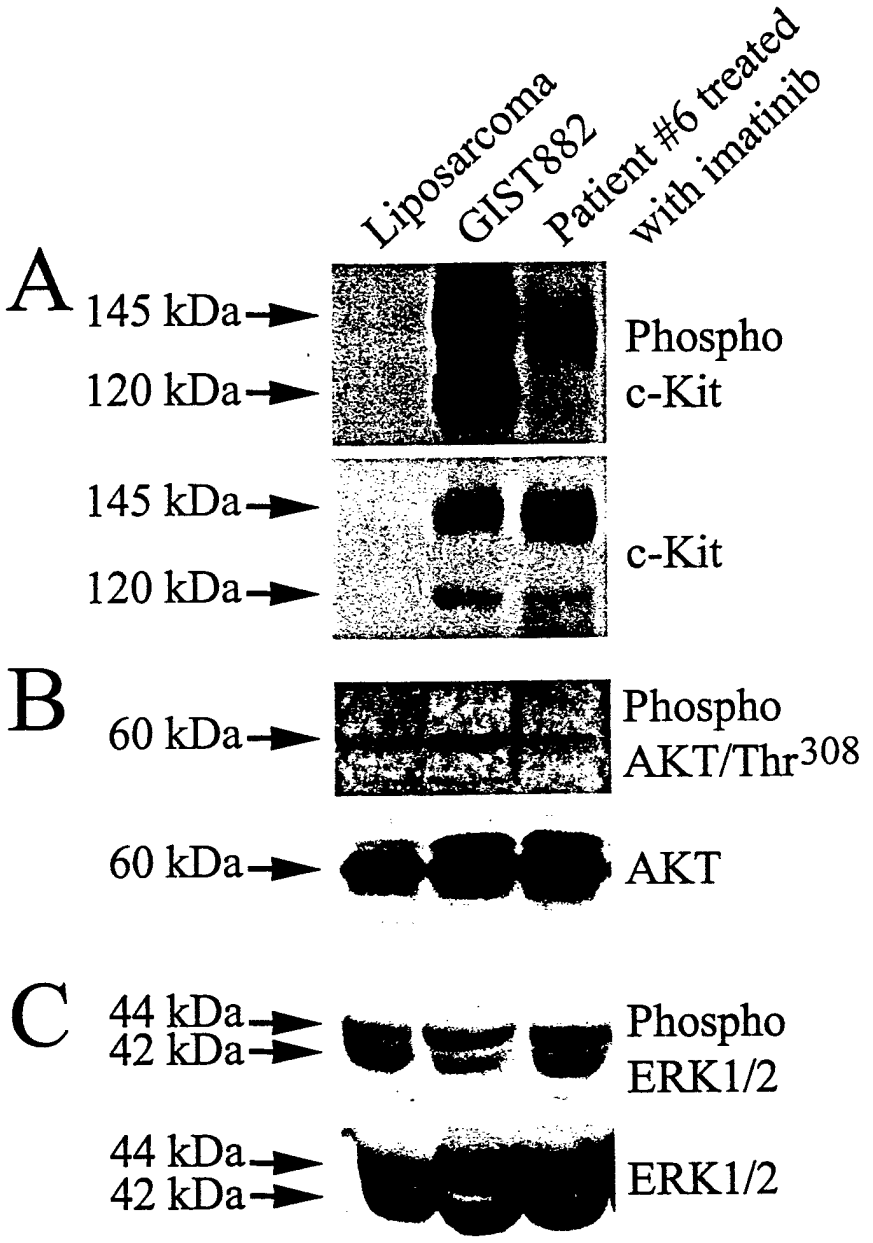

Imitanib 10 μM
Hours
0 6 12 24 48
28s rRNA
SPRY4A
28s rRNA
18s rRNA
EtBr


Imatinib 10 μM
primers 1F/1R
primers 2F/2R
M 0 6 12 24 48 0 6 12 24 48 (hr)
bp
850
600
500
200
SPRY4A
SPRY4A/4C
SPRY4C
1000
850
β-actin


A
Imatinib 1 μM
Imatinib 10 μM
Hours 0 0.5 1 3 6
0 0.5 1 3 6
145 kDa
120 kDa
Phospho c-Kit
145 kDa
120 kDa
c-Kit
60 kDa
Phospho AKT/Thr[3]
60 kDa
AKT
44 kDa
42 kDa
Phospho ERK1/2
44 kDa
42 kDa
ERK1/2
B
SPRY4A
β-actin


A
U0126 30 μM
Hours 0 0.5 1 3 6
Phospho ERK1/2
ERK1/2
AKT/Thr308
AKT
B
SPRY4A
β-actin


#1 (R)
#2 (R)
#3 (R)
#4 (R-P)
#5 (NR)
#6 (NR)
Imatinib
− + − + − + − + + − + − +
SPRY4A
β-actin


Liposarcoma
GIST882
Patient #6 treated with imatinib
A
145 kDa
120 kDa
Phospho c-Kit
145 kDa
120 kDa
c-Kit
B
60 kDa
Phospho AKT/Thr308
60 kDa
AKT
C
44 kDa
42 kDa
Phospho ERK1/2
44 kDa
42 kDa
ERK1/2

Int. J. Cancer: 99, 185–192 (2002)

Publication of the International Union Against Cancer

OVCA2 IS DOWNREGULATED AND DEGRADED DURING RETINOID-INDUCED APOPTOSIS

Amanda H. PROWSE[1]*, Lisa VANDERVEER[1], Simon W.F. MILLING[3], Zhong-Zong PAN[1], Roland L. DUNBRACK, JR.[1], Xiang-Xi XU[1] and Andrew K. GODWIN[1,2]

[1]*Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA*
[2]*Human Genetics Program, Fox Chase Cancer Center, Philadelphia, PA, USA*
[3]*Department of Cell and Developmental Biology, Fox Chase Cancer Center, Philadelphia, PA, USA*
[4]*Basic Science Division, Fox Chase Cancer Center, Philadelphia, PA, USA*

Retinoids, the natural and synthetic derivatives of vitamin A, have been shown to regulate the growth and differentiation of a wide variety of cell types and consequently have enormous potential as chemotherapeutic agents. We have previously identified 2 genes, termed *OVCA1* and *OVCA2*, which are located in a small region showing a high frequency of allelic loss in breast and ovarian tumors and share a common exon. Recent studies have suggested that expression of OVCA1 may be influenced by retinoids. Therefore, we analyzed the expression of OVCA1 and OVCA2 in cells in response to treatment with all-trans retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4HPR), or under conditions of low serum and confluence, to determine further the roles of OVCA1 and OVCA2 in cell growth, apoptosis and differentiation. We show that OVCA2 mRNA and protein are ubiquitously expressed and that they are downregulated in the lung cancer cell line Calu-6 after treatment with RA and 4HPR. In addition, we observed that OVCA2 protein is proteolytically degraded in response to RA and 4HPR treatment in a time- and dose-dependent manner in the promyelocytic leukemia cell line HL60. In contrast, expression of the candidate tumor suppressor OVCA1 was not downregulated by these treatments. Furthermore, we demonstrate that OVCA2 is evolutionarily conserved and shows regional homology with dihydrofolate reductases (DHFRs), specifically with hydrolase folds found in α-β hydrolases. Our results are in contrast to a previous report and show that OVCA2, not OVCA1 mRNA and protein, is downregulated in response to RA and 4HPR.

Key words: *OVCA1; OVCA2; all-trans retinoic acid; RA; N-(4-hydroxyphenyl)retinamide; 4HPR*

Retinoids, the natural and synthetic derivatives of vitamin A, have been shown to regulate the growth and differentiation of a wide variety of cell types and consequently have enormous potential as chemotherapeutic agents.[1,2] The diverse effects of retinoids are mediated by binding to at least 6 retinoid receptors, which fall into 2 subfamilies: retinoic acid receptors (RARs) α, β and γ and the retinoid X receptors (RXRs) α, β and γ.[3] The RARs and RXRs act as transcription factors, binding as homo- and heterodimers to retinoid response elements in the promoter regions of target genes and thus enhancing or repressing transcription. In addition, RARs and RXRs can inhibit the expression of AP1-dependent genes by antagonizing AP1 activity.[4–6] However, many of the downstream targets that lead to retinoid-induced growth arrest, differentiation and/or apoptosis remain to be identified. In addition, synthetic retinoids, such as 4HPR, which have been developed as chemoprevention agents with an acceptable toxicity profile, may well differ in their mechanism of action.[7–9]

Loss of heterozygosity (LOH) at 17p13.3 has been reported in ovarian tumors, breast tumors, primitive neuroectodermal tumors, carcinoma of the cervix uteri, medulloblastomas and lung tumors, suggesting that genes on 17p13.3 may play a role in the development of multiple cancers.[10–18] We and others have previously defined a minimum region of allelic loss (MRAL) on chromosome 17p13.3 in genomic DNA from ovarian tumors.[19,20] Positional cloning and sequencing techniques revealed at least 2 candidate tumor suppressor genes in the ~20 kb MRAL, referred to as *OVCA1* or *DPH2L* and *OVCA2*.[19,20] The *OVCA1/DPH2L* and *OVCA2* genes overlap one another in the MRAL and have 1 exon in common.[20] Since translation of *OVCA1/DPH2L* does not proceed into the shared exon (exon 13 in *OVCA1/DPH2L* and exon 2 in *OVCA2*), the genes encode for completely distinct OVCA1 and OVCA2 proteins.

We have previously shown that *OVCA1* is a strong candidate for a tumor suppressor gene: it is downregulated in a proportion of breast and ovarian tumors, and overexpression of *OVCA1* reproducibly inhibits colony formation in a variety of tumor cell lines.[21] However, recent studies have suggested that OVCA1 may be downregulated after differentiation or growth arrest induced by RA in lung cancer cell lines, contrary to a role as a tumor suppressor.[22] Therefore, we have analyzed both OVCA1 and OVCA2 mRNA and protein expression in cell lines treated with all-trans retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4HPR), or under conditions of low serum and confluence, to determine a possible role of OVCA1 and/or OVCA2 in cell growth, apoptosis and differentiation. We show that OVCA2 mRNA and protein is downregulated in the lung cancer cell line Calu-6 treated with RA and 4HPR, but that OVCA1 is unaffected. Interestingly, OVCA2 protein is degraded in response to RA and 4HPR treatment in the promyelocytic leukemia cell line HL60. In addition, we present a further characterization of *OVCA2* and show that it is a highly conserved gene that is related to a variety of α-β hydrolases including esterases, lipases and other enzymes.

MATERIAL AND METHODS

Cell lines

Cos-1, MCF-7, SKOV-3, HeLa and A2780 cells were maintained in DMEM supplemented with 10% FCS, glutamine and insulin. A549 cells were maintained in Kaighn's modification of Ham's F12 medium supplemented with 10% FCS and glutamine. HL-60 cells and Calu-6 cells were maintained in RPMI-1640 supplemented with 10% FCS and glutamine. Human ovarian surface epithelial cell lines expressing SV-40 large T-antigen (HIO cells) have been previously described.[23]

Grant sponsor: National Institutes of Health; Grant number: RO1 CA-70328; Grant sponsor: United States Army Medical Research; Grant number: DAMD17-96-1-6088; Grant sponsor: Ovarian Cancer Research Fund; Grant sponsor: Eileen Stein Jacoby Fund; Grant sponsor: American Cancer Society; Grant sponsor: Commonwealth of Pennsylvania.

*Correspondence to: Department of Medical Oncology, Room w322, Fox Chase Cancer Center, 7701 Burholme Ave, Philadelphia, PA 19111, USA. Fax: +215-728-2741. E-mail: AH_Prowse@fccc.edu

Received 20 July 2001; Revised 30 November 2001; Accepted 11 January 2002

DOI 10.1002/ijc.10334

Cell culture treatments

RA (Sigma, St. Louis, MO) and 4HPR (NIH) were dissolved in ethanol at a stock concentration of 10 mM. Calu-6 and A549 cells were seeded at equal density (5×10^5 cells) in 100 mm dishes and allowed to attach for 24 hr. They were then treated with either 10 and 1 μM RA or 4HPR or ethanol alone for 24, 48, 72 or 96 hr. HL60 cells, which are nonadherent, were seeded into T25 flasks at equal density (1×10^5 cells) and treated with 1 or 0.1 μM RA and 4HPR or ethanol alone for 24, 48, 72 and 96 hr. A549 cells were cultured in 1% FCS for 1 week or cultured for 3 days after confluence.

Preparation of RNA and protein extracts

Total cellular RNA was isolated using guanidinium/isothiocyanate/phenol/chloroform as previously described.[20] Whole cell protein extracts were made by incubating cells in PBSTDS (10 mM Na_2HPO_4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.2% NaN_3, 1 mM EDTA, 5 mM NaF, 100 μg/ml PMSF, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin, pH 7.25) as described previously.[21] Quantitation of protein was determined using a bicinchoninic acid/copper (II) sulfate assay (Sigma). Extracts from normal human tissues were purchased from ClonTech (Palo Alto, CA).

Northern blot analysis

Fifteen micrograms of total RNA was used for standard Northern blot analysis, as previously described.[20] Multiple tissue Northern blots containing 5 μg of poly(A)$^+$-selected mRNA from various human tissues were purchased from Clontech. Blots were hybridized with a ~830 bp cDNA probe corresponding to exon 13 of *OVCA1* and exon 2 of *OVCA2* or a ~200 bp cDNA probe corresponding to exon 1 of *OVCA2*.

Antibodies

Anti-β-actin was purchased from Sigma. Anti-PARP antibody was purchased from Cell Signaling (Beverly, MA). The anti-OVCA1 antibody TJ132 has been described previously.[21] For the production of anti-OVCA2 antibodies, a peptide corresponding to amino acids 27–41 of OVCA2 was synthesized (Research Genetics, Huntsville, AL). Purity of the peptide was confirmed by high-performance liquid chromatography. The peptide was conjugated to malemide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) and used to immunize a New Zealand White rabbit (Cocalico Biologicals, Reamstown, PA). Two milligrams of antigenic peptide were covalently linked to Aminolink agarose (Pierce) and used to purify anti-OVCA2 antibody according to the manufacturer's instructions. The final antibody was referred to as TJ143.

Caspase-3 activity assay

Colorimetric CaspACE assay (Promega, Madison, WI) was used to detect caspase-3 activity. HL60 cells treated with 1 or 0.1 μM RA or 4HPR for 24, 48, 72 and 96 hr or with ethanol alone were harvested, snap-frozen and stored at −70°C. The frozen cell pellet was lysed (20 μl lysis buffer per million cells) by 1 cycle of freezing and thawing. Twenty micrograms of cell lysate (20–50 μg total protein) was incubated with the caspase-3 substrate Ac-DEVD-pNA for 4 hr at 37°C. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate.

FACS analysis

HL60 cells treated with 1 μM RA or 4HPR or ethanol alone for 96 hr were harvested and stained with propidium iodide. Fluorescent cells were analyzed using a FACScan machine running CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The percentage of cells in each stage of the cell cycle was calculated by the Watson Pragmatic algorithm, using FlowJo software (Tree Star, San Carlos, CA).

cDNA cloning and cell transfections

Cloning of the full-length *OVCA2* cDNA and genomic DNA was previously described.[20] Genomic *OVCA2* DNA was subcloned into the mammalian expression vector pcDNA3 (InVitrogen, La Jolla, CA) by PCR-amplifying a DNA fragment using gene-specific primers containing *Bam*HI (5′) or *Eco*RI (3′) restriction endonuclease sites, digesting the fragment and cloning it into the multiple cloning sequence of pcDNA3. To produce an N-terminal hemagglutinin (HA)-tagged OVCA2 expression vector, *OVCA2* cDNA was first cloned into the *HA*-containing mammalian expression vector J3H, and then the *HA-OVCA2* cDNA was subcloned into pcDNA3. Cell lines were transfected during the log phase of growth with 5 μg of vector using the Superfect reagent (Qiagen, Chatsworth, CA), according to the manufacturer's instructions.

SDS-PAGE and Western blot analysis

Fifty micrograms of total protein extract from tissues or 30 μg of total protein from cell extracts, unless otherwise stated, were separated by standard SDS-PAGE and transferred to Immobilon-P (Millipore, Bedford, MA). The membranes were blocked either in 3% BSA and probed with anti-OVCA1 antibody TJ132 or in 5% dried milk and probed with the anti-OVCA2 antibody TJ143 or the anti-β-actin antibody (Sigma).

Sequence analysis

GenBank/EMBL and SwissProt sequences showing homology to OVCA2 were identified using the basic local alignment search tool (BLAST, NCBI). DNA and amino acid sequence comparisons and motif analyses were performed with the Wisconsin Package versions 8 and 9.1 (Genetics Computer Group).

Homology modeling of OVCA2

We used methods described previously to build a model of OVCA2.[24,25] Briefly, PSI-BLAST was used to build a sequence profile of OVCA2 by iteratively searching the nonredundant protein sequence database available from the NCBI.[26] Only sequences with expectation values better than 0.0001 were included in the sequence profile matrix. Upon completion, this matrix was used to search a database of protein sequences in the Protein Data Bank[27] of experimentally determined protein structures. A model of OVCA2 was built using the side-chain conformation prediction program SCRWL,[28] which works by building side chains on a template backbone by first placing residues according to a backbone-dependent rotamer library,[29] followed by a combinatorial search to remove steric overlaps.

RESULTS

Analysis of OVCA1 and OVCA2 under conditions of growth arrest and apoptosis

We have previously reported the isolation of 2 candidate tumor suppressor genes, referred to as *OVCA1* and *OVCA2*.[20] A schematic of the 2 genes is shown in Figure 1. The full cDNA sequence of *OVCA1* and *OVCA2* has been deposited into GenBank (accession numbers AF335321 and AF321875, respectively). Recent studies suggested that *OVCA1* was downregulated in response to cell differentiation, growth arrest and apoptosis induced by RA and 4HPR in the lung cancer cell lines Calu-6 and GLC-82 and the promyelocytic leukemia cell line HL60.[22] Since the results were in sharp contrast to our previous findings of dramatic growth suppression induced by overexpression of OVCA1, we elucidated the expression of OVCA1 and OVCA2 in response to RA and 4HPR. Initially we analyzed the lung cancer cells Calu-6 and A549, using the same treatments described by Liu and colleagues,[22] *i.e.*, cells were treated with 10 μM RA and 4HPR for a period of 4 days.

To determine whether the RA and 4HPR treatments were affecting the growth properties of our cell lines, we performed direct cell counts (Fig. 2*a*). Both cell lines treated with RA and 4HPR exhibited a decrease in cell number compared with control (Fig. 2*a*). We analyzed the expression of *OVCA1* and *OVCA2* after RA and 4HPR treatment by Northern blot analysis using a probe to exon 2/13 of *OVCA1/2* (Fig. 1). As shown in Fig. 2*b*, ≃2.4 and 1.3 kb transcripts were expressed in both cell lines, corresponding to *OVCA1* and *OVCA2* respectively.[20] The 1.3 kb *OVCA2* transcript

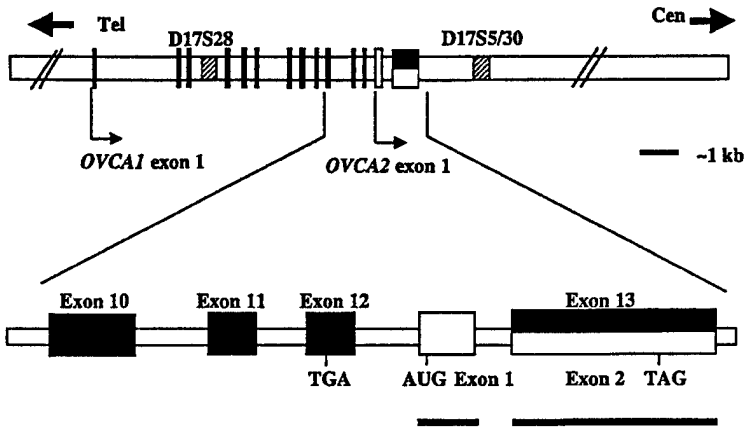


FIGURE 1 – Genomic organization of *OVCA1* (exons shown in black boxes) and *OVCA2* (exons shown in white boxes). *OVCA1* and *OVCA2* map to a 20 kb minimum region of allelic loss in ovarian tumors, between the markers D17S28 and D17S5/30, at 17p13.3.[20] The *OVCA1* and *OVCA2* genes overlap one another and have 1 exon in common (exon 13 of *OVCA1* and exon 2 of *OVCA2*). Since translation of *OVCA1* does not proceed into exon 13 in *OVCA1* or exon 2 in *OVCA2*, the genes encode for completely distinct OVCA1 and OVCA2 proteins. Probes used for Northern hybridizations are shown.

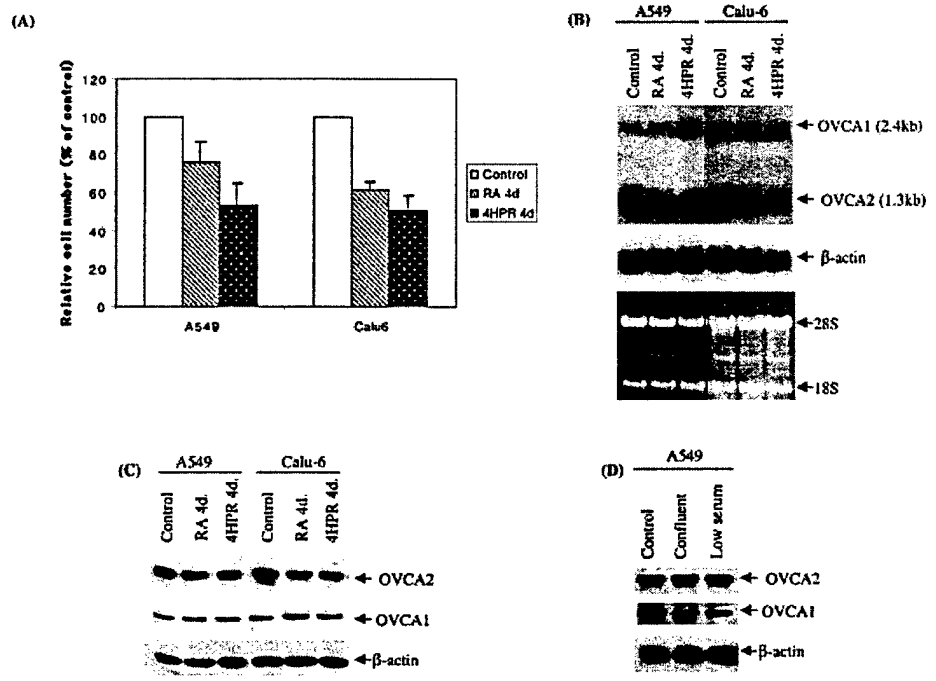


FIGURE 2 – (*a*) Growth inhibition of cells treated with all-trans retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4HPR). Cells treated with 10 μM RA or 4HPR for 4 days were harvested and stained for trypan blue uptake, and unstained cells were counted using a hemocytometer. The mean relative cell number as a percentage of the control is shown for both cell lines from 3 separate experiments, together with the standard deviation, shown as error bars. (*b*) Analysis of *OVCA2* and *OVCA1* mRNA expression in Calu-6 and A549 cells treated with 10 μM RA or 4HPR for 4 days compared with control cells (ethanol alone). Fifteen micrograms of total RNA from the indicated cell lines and treatments were used for standard Northern blot analysis. The Northern blot was hybridized with a probe corresponding to exon 13 of *OVCA1* and exon 2 of *OVCA2*, or with a probe to β-actin, as a control, as indicated. Lower panel: Ethidium bromide-stained gel prior to blotting. The position of the 28S and 18S rRNA is indicated. (*c*) Analysis of OVCA2 and OVCA1 protein expression in Calu-6 and A549 cells treated with 10 μM RA and 4HPR compared with control cells (ethanol alone) for 4 days. Thirty micrograms of extracts from the indicated cell lines and treatments were separated by 12% SDS-PAGE and processed by Western blotting. The blots were probed with the anti-OVCA2 antibody TJ143 or the anti-OVCA1 antibody TJ132. Anti-β-actin was used a loading control. A representative figure is shown from 2 separate experiments. (*d*) Analysis of the effects of confluence and low serum on OVCA2 and OVCA1 expression. A549 cells were growth-arrested by culturing in 1% serum for 1 week or for 3 days after confluence. Thirty micrograms of extracts from the indicated treatments were separated by 12% SDS-PAGE and processed by Western blotting. The blots were probed with the anti-OVCA2 antibody TJ143 or the anti-OVCA1 antibody TJ132. Anti-β-actin was used a loading control. A representative figure is shown from 2 separate experiments.

was downregulated in both Calu-6 and A549 cells in response to RA and 4HPR treatment. Liu *et al.*[22] also found that a ~1.7 kb transcript was reproducibly downregulated in Calu-6 cells after a 4-day treatment with RA or 4HPR; however, the authors interpreted the ~1.7 kb transcript to be a smaller transcript of *OVCA1/DPH2L*. The 2.3 kb *OVCA1* transcript was unaffected by RA treatment in both cell lines. We did observe slight variations (<1-fold) in OVCA1 mRNA levels in A549 and Calu-6 cells

treated with 4HPR; however, β-actin normalized levels were unchanged (Fig. 2*b*).

To clarify further the effect of RA and 4HPR on OVCA1 and OVCA2, we analyzed their protein expression after 4-day treatment with 10 μM RA or 4HPR by Western blot analysis. Antibodies against amino acids 27–41 of OVCA2 were generated by injecting the peptide into rabbits, and the antiserum was immunoaffinity purified (referred to as TJ143). The anti-OVCA1 antibodies TJ132 have been described previously.[21] In Calu-6 cells OVCA2 protein levels were downregulated by RA and 4HPR, but OVCA1 was not significantly affected (Fig. 2*c*). In A549 cells, neither OVCA2 nor OVCA1 protein levels were measurably affected by RA or 4HPR treatment (Fig. 2 *c*). Furthermore, OVCA2 protein levels were unaffected by growth arrest induced by cell confluence for 3 days or low serum for 1 week in A549 cells, whereas OVCA1 levels were decreased >2-fold in serum-deprived cells (Fig. 2*d*).

The effect of RA and 4HPR on OVCA1 and OVCA2 protein levels in Calu-6 and A549 cells was further evaluated. Cells were treated with 1 or 10 μM RA or 4HPR for 24, 48, 72 or 96 hr. In Calu-6 cells, OVCA2 protein only appeared to be downregulated after 96 hr of treatment with 10 μM RA and after 48 hr of treatment with 10 μM 4HPR (Fig. 3). OVCA1 protein was not affected by these treatments in Calu-6 cells. In A549 cells, OVCA2 was slightly downregulated after 48, 72 and 96 hr of treatment with 10 μM RA and 4HPR. OVCA1 protein levels appeared to increase after 96 hr of treatment with 10 μM 4HPR; however, this increase was not always consistently observed. Neither OVCA1 nor OVCA2 were affected by the lower dose of 1 μM RA and 4HPR during these time points in either cell line (data not shown). Overall, the results suggest that, in contrast to the conclusions from a previous report, OVCA2, not OVCA1 can be downregulated in response to RA and 4HPR treatment.

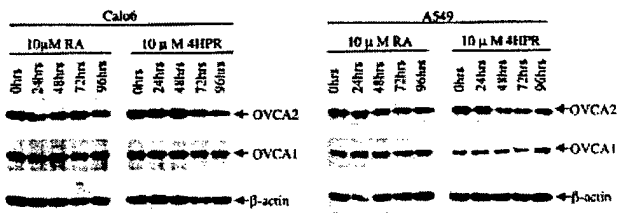


FIGURE 3 – Analysis of OVCA2 and OVCA1 protein expression in Calu-6 and A549 cells lines treated with 10 μM all-trans retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4HPR) compared with control cells (ethanol alone) for 24, 48, 72 and 96 hr. Thirty micrograms of extracts from the indicated cell lines and treatments were separated by 10% SDS-PAGE and processed by Western blotting. The blots were probed with the anti-OVCA2 antibody TJ143 or the anti-OVCA1 antibody TJ132. Anti-β-actin was used a loading control.

OVCA2 is degraded in HL60 cells in response to RA and 4HPR treatment

We also analyzed the effect of RA and 4HPR on OVCA2 expression in HL60 cells. Previous studies have shown that in HL-60 cells RA promotes differentiation, followed by apoptosis, and that 4HPR induces apoptosis.[30,31] Cells were treated with 0.1 and 1 μM RA and 4HPR, and the mRNA and protein levels were evaluated by Northern and Western blotting, respectively. As expected, the mRNA levels were reduced >2-fold (data not shown). Interestingly, the OVCA2 protein levels were dramatically reduced in response to RA and 4HPR treatments (Fig. 4*a*). The level of 25 kDa full-length protein appeared to correlate directly with the length of treatment and the drug dosage of both RA and 4HPR (Fig. 4*a*). HL60 cells are nonadherent, and therefore this may be an indication of the number of dead cells, which, in contrast to attached cell lines such as Calu-6 and A549, are not removed during refeeding and when harvested. Thus, the protein degradation may be a result of RA- and 4HPR-induced apoptosis. To determine whether the HL60 cells were indeed undergoing apoptosis, we performed FACS analysis and analyzed caspase 3 activity. We did not see an increase in caspase 3 activity as assessed using a Colorimetric CaspACE assay and PARP cleavage (data not shown). However, the FACS analysis did show an increase in the sub G_1/G_0 fraction, indicating increased cell death, as well as a dramatic arrest in G_1/G_0 (Fig. 4*b*). These results

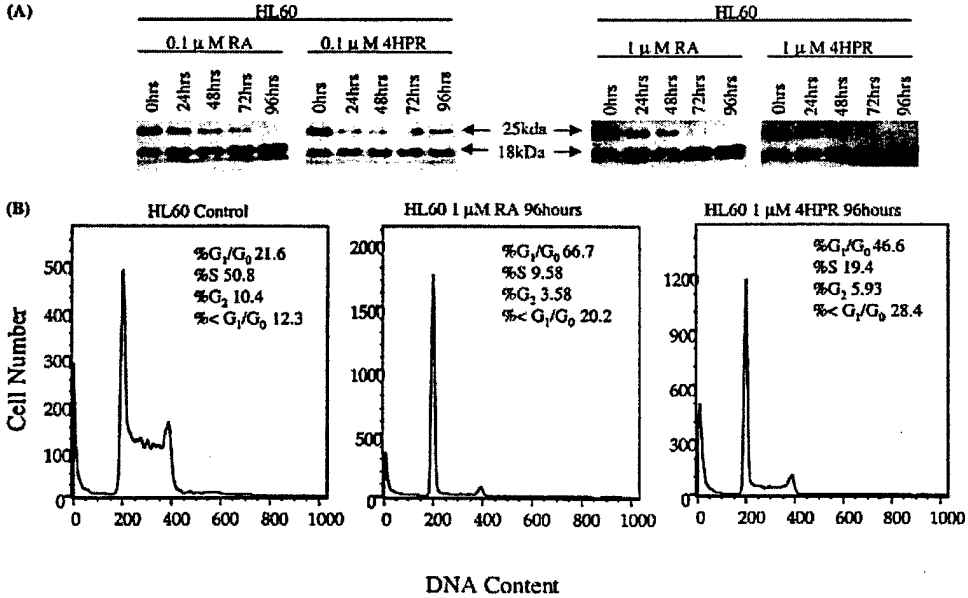


FIGURE 4 – (*a*) Analysis of OVCA2 protein expression in HL60 cells treated with 0.1 μM and 1 μM all-trans retinoic acid (RA) and N-(4-hydroxyphenyl)retinamide (4HPR) compared with control cells (ethanol alone) for 24, 48, 72 and 96 hr. Fifty micrograms of extracts from the indicated treatments were separated by 10% SDS-PAGE and processed by Western blotting. The blots were probed with the anti-OVCA2 antibody TJ143. (*b*) FACS analysis of HL60 cells treated with 1 μM RA or 4HPR or ethanol alone for 96 hr. Treated cells were harvested and stained with propidium iodide and analyzed by FACS. The percentage of cells in each stage of the cell cycle is indicated.

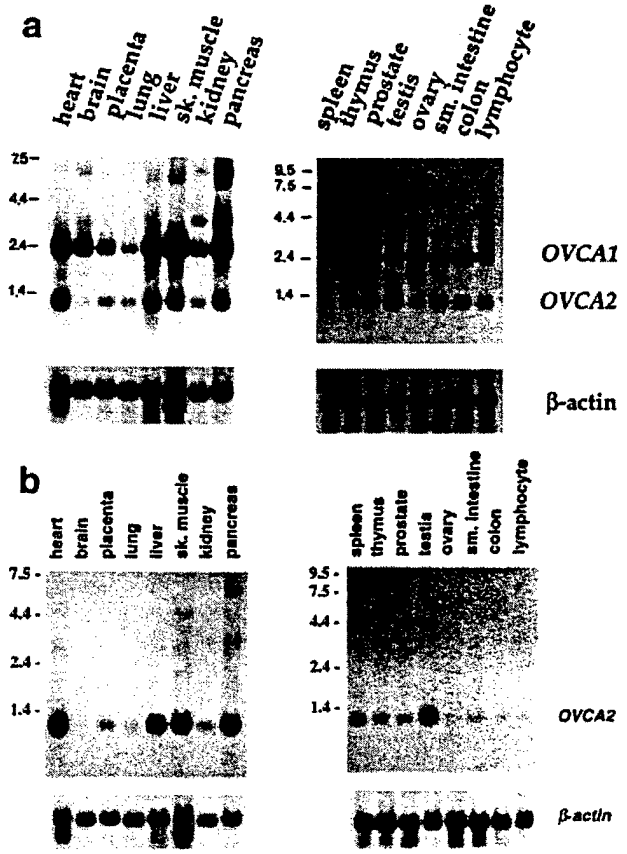


FIGURE 5 – Tissue expression pattern of *OVCA1* and *OVCA2* mRNA. Blots containing 5 μg of polyA$^+$ selected mRNA from each of the indicated human tissues were hybridized with a ~830 bp cDNA probe corresponding to exon 13 of *OVCA1* and exon 2 of *OVCA2* (*a*) or a ~200 bp cDNA probe corresponding to exon 1 of *OVCA2* (*b*). Size standards are in kilobases.

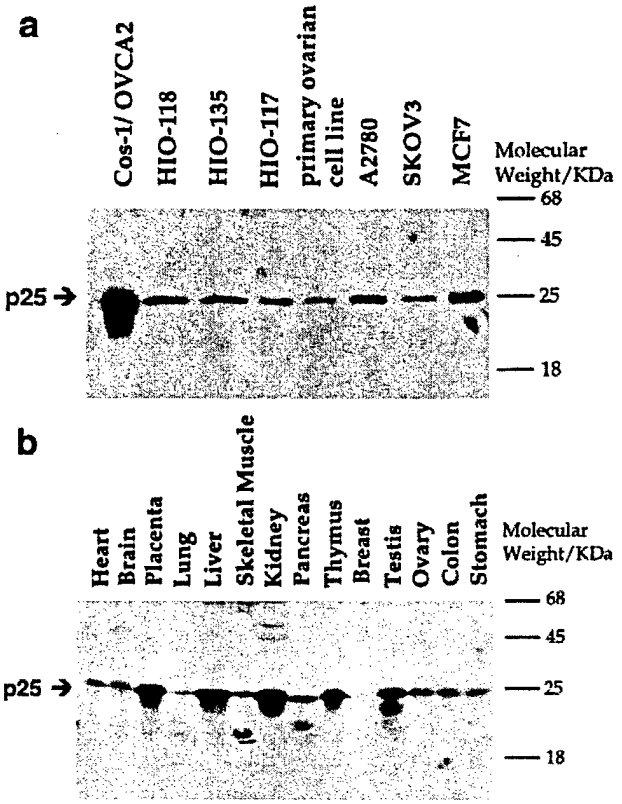


FIGURE 6 – (*a*) Characterization of OVCA2 expression. Twenty micrograms of extracts from the indicated cell lines were separated by 12% SDS-PAGE and processed by Western blotting. The blot was probed with the anti-OVCA2 antibody, TJ143. Lane Cos-1/*OVCA2*, extract of Cos-1 cells that had been transfected with pcDNA3-OVCA2; lanes HIO-118, HIO-135, HIO-117, extracts from SV40 Tag-immortalized human ovarian surface epithelial cell lines (HIO); lane primary ovarian cell line, extract from human ovarian surface epithelial cell line; lanes A2780 and SKOV3, extracts from ovarian cancer cell lines; lane MCF-7, extract from breast cancer cell line. (*b*) OVCA2 expression in human tissues. Fifty micrograms of extracts from the indicated human tissues (Clontech) were separated by 12% SDS-PAGE and processed by Western blotting. The blot was probed with the anti-OVCA2 antibody TJ143.

suggest that OVCA2 may be targeted for protein degradation after RA- or 4HPR-induced differentiation and/or apoptosis.

OVCA2 *has a broad tissue distribution*

To characterize *OVCA2* further, we analyzed *OVCA2* mRNA expression in a variety of tissues. Multiple tissue Northern blots were probed with the exon 2/13 of *OVCA1/2* or the unique exon 1 of *OVCA2*, as depicted in Figure 1. As shown in Figure 5*a*, all tissues exhibited 2 bands (~2.4 and 1.3 kb). When our blots were reprobed with an exon 1 probe of *OVCA2*, all tissues tested exhibited only the 1.3 kb band representing the *OVCA2* transcript, with testis, heart, skeletal muscle, liver and pancreas showing high mRNA expression (Fig. 5*b*).

In addition, we analyzed OVCA2 protein expression in a number of cell lines and tissues. Western blot analysis using the anti-OVCA2 antibody TJ143 revealed that Cos-1 cells, transfected with a genomic DNA fragment containing the 2 exons of OVCA2 under the control of a CMV promoter, produced the predicted ~25 kDa protein (Fig. 6*a*). The same results were obtained when Cos-1 cells were transfected with the OVCA2 cDNA (not shown), indicating that the mRNA transcribed from the genomic DNA was correctly spliced within the cells. The antibody also detected endogenous OVCA2 in various breast and ovarian cell lines (Fig. 6*a*) and in a variety of human tissues (Fig. 6*b*). Of the tissues analyzed, the kidney, liver, testis, placenta and thymus all exhibited high levels of OVCA2 (Fig. 6*b*).

OVCA2 is highly evolutionarily conserved

The OVCA2 protein consists of 227 amino acids (Fig. 7). A BLAST search of GenBank/EMBL and SwissProt databases revealed that *OVCA2* does not match any known mammalian genes. However, 1 *C. elegans* and 4 yeast proteins were identified that exhibited up to 60% similarity and up to 45% identity to the amino acid sequence of OVCA2 and that contained a similar number of amino acids (Fig. 7). These sequences were described as putative dihydrofolate reductases (DHFRs), but they share more conserved domains with OVCA2 than with mammalian DHFRs (data not shown). A BLAST search of the EST database revealed full-length mouse and partial rat OVCA2 homologs displaying 87% and 86% similarity, respectively, to the amino acid sequence of OVCA2. In addition, 2 plant ESTs (rice and arabidopsis; up to 53% similar) and multiple human sequences were identified. A multiple sequence alignment of OVCA2 with all available non-human OVCA2 homologs (Fig. 7) revealed at least 5 conserved domains, which presently have no known function, but which may be important new functional domains based on their evolutionary conservation. Zoo blots probed with the unique exon 1 of *OVCA2* demonstrated that all mammalian species tested have an *OVCA2* homolog (data not shown). Interestingly, when exon 2 of *OVCA2*, which is a noncoding exon of *OVCA1*, was used to probe these

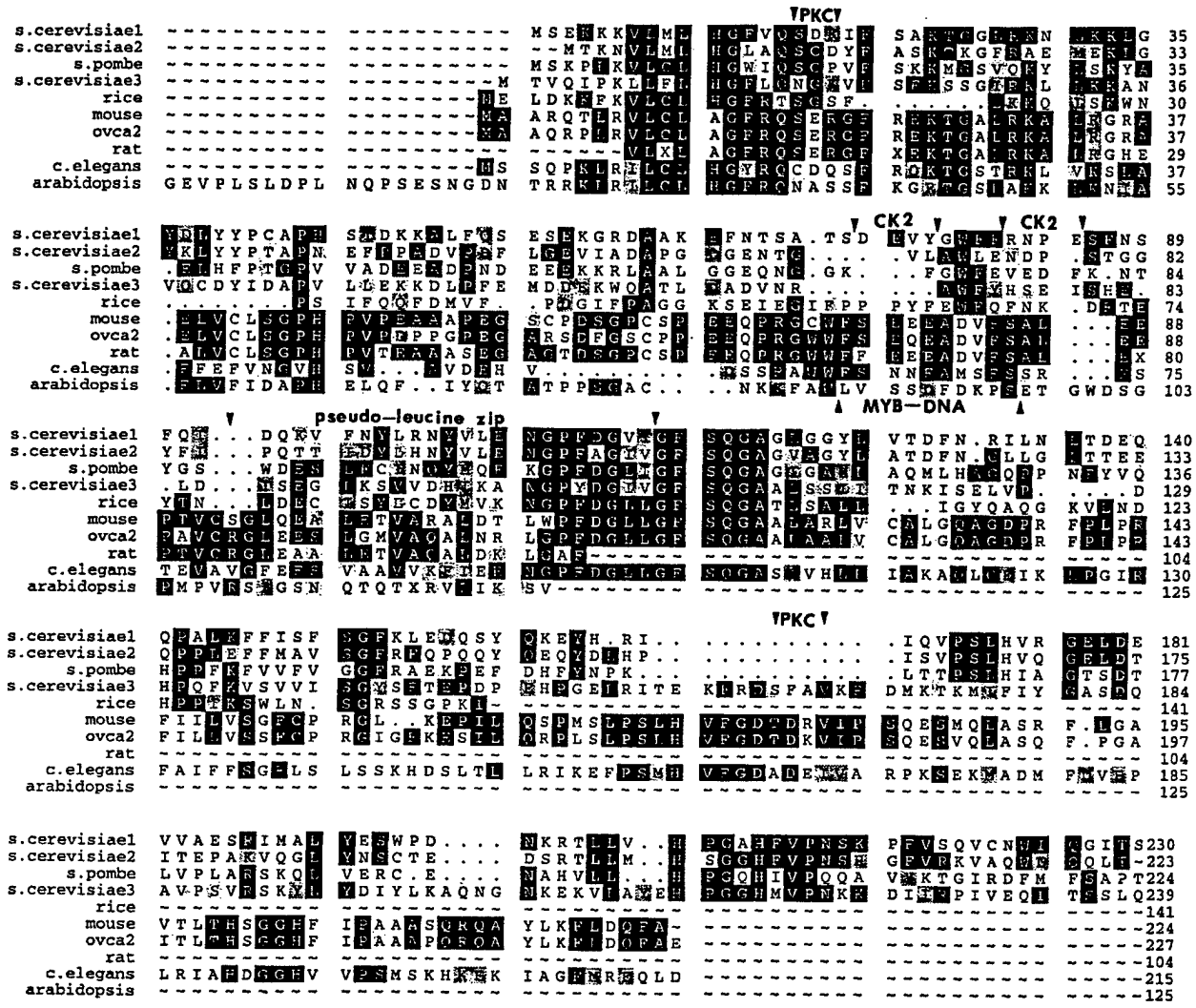


FIGURE 7 – Multiple sequence alignment of OVCA2 amino acid sequence and similar sequences from mouse, rat, *C. elegans*, *S. cerevisiae*, *S. pombe*, rice and arabidopsis. At least 5 conserved regions are evident. Two protein kinase C phosphorylation sites (PKC), 2 casein kinase-2 sites (CK2), a potential pseudo-leucine zipper motif and a potential MYB DNA binding site (MYB-DNA) are all indicated.

blots, both *OVCA2* and *OVCA1* bands were identified, suggesting that the genomic arrangement of the 2 genes is conserved among many different species. This high degree of evolutionary conservation suggests that *OVCA2* may be very important for normal cellular function.

The Genetics Computer Group package was used to evaluate functional motifs within the OVCA2 amino acid sequence (Fig. 7). Two protein kinase C phosphorylation sites (amino acids 18 and 178), 2 casein kinase-2 phosphorylation sites (amino acids 76 and 84) and a possible leucine zipper variant (amino acid 95) were identified, all of which are conserved within the available mouse and rat sequences. In addition, a MYB DNA binding motif was observed (amino acid 83), which is identical to the native MYB motif, except for a conservative amino acid change from tryptophan to phenylalanine. Interestingly, this domain contains 1 of the casein kinase-2 phosphorylation sites. No other functional groups were identified that could provide clues to the function of OVCA2.

OVCA2 protein model

Sequence analysis using PSI-BLAST indicated that OVCA2 is related to the N-terminal domain of some DHFRs, notably DYR_SCHPO, a DHFR in yeast. This domain has a hydrolase fold that is found in α-β hydrolases including esterases and lipases and includes the 3 residues of the catalytic triad, Asp, Ser, His (Fig. 8). The crystal structure of Protein Data Bank entry 1AUR[32] was used to build a model of OVCA2 using the side chain conformation prediction program SCWRL.[28] The sequence identity between OVCA2 and the crystal structure is only 13%, but the same fold was identified with high confidence with 3 different programs, PSI-BLAST,[26], 3d-pssm[33] and Threader.[34] The significance of this domain within the DHFRs, however, has yet to be reported.

DISCUSSION

We have found that expression of OVCA2 but not OVCA1, is downregulated in cells in response to RA and 4HPR. This is in contrast to a recent paper by Liu *et al.*,[22] in which it was reported that *OVCA1/DPH2L* mRNA levels were decreased in several cancer cell lines after treatment with RA or 4HPR. The authors used mRNA differential display to uncover genes modulated by RA in human lung cancer cell lines, and a clone was identified that was homologous to the 3′UTR of *OVCA1/DPH2L*. They performed Northern blot analysis with a probe to a 3′ fragment of *OVCA1/DPH2L* that detected both a ~2.3 kb trancript and a ~1.7

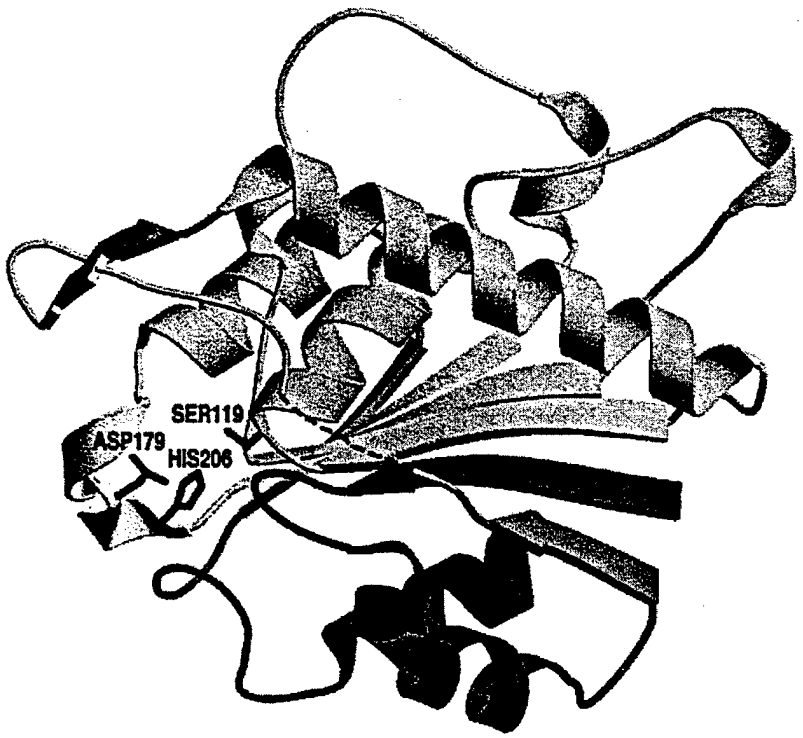


FIGURE 8 – Molecular model of OVCA2 shown as a ribbon diagram. The 3 residues of the catalytic triad conserved in α-β hydrolases are shown as stick figures. The model was built from Protein Data Bank entry 1AUR.[31]

kb transcript, which is consistent with our Northern blot data when probed with exon 2/13 of *OVCA1/2*. We[20] and Phillips *et al.*[35] have previously described the 2.4 kb/2.3 kb transcript as *OVCA1/DPH2L*. However, Liu and colleagues[22] interpreted the ~1.7 kb transcript to be a smaller transcript of *OVCA1/DPH2L*. We have now clarified that the 1.7 kb/1.3 kb transcript encodes for OVCA2, which is an entirely different protein from OVCA1/DPH2L.

We have found that the OVCA2 mRNA is downregulated in response to 10 μM RA and 4HPR in the lung cancer cell lines A549 and Calu-6. Liu and colleagues[22] also found that the 1.7 kb transcript, which we have now shown corresponds to *OVCA2*, is downregulated in Calu-6 cells and slightly downregulated in A549 cells. We have evaluated the sequence of the *OVCA2* promoter for potential retinoic acid response elements (RAREs) and AP1 binding sites. Our initial screen of the first 10 kbp of genomic sequence upstream of exon 1 failed to uncover any of the common RARE consensus sequences.[36] However, we did identify 3 putative AP1 binding sites, and it is possible that OVCA2 may be downregulated because retinoid receptors antagonize AP1 activity.[6]

At the protein level, which is a more relevant biologic marker for cellular processes, the effect on OVCA2 in A549 cells appears to be less dramatic, and therefore there must be some compensation in OVCA2 expression at the posttranscriptional level. Indeed, there are also differences in mRNA and protein levels seen in the same tissue type from the multiple tissue Northern and Western blots, although the tissue sources are different and therefore it is difficult to do a direct comparison. In Calu-6 cells, OVCA2 protein was downregulated after RA and 4HPR treatment; however, this only occurred with high doses (10 μM), and after 3–4 days. The most dramatic and interesting effect was seen with HL60 cells in which OVCA2 was degraded in response to RA- and 4HPR-induced cell differentiation and apoptosis. It has been shown that HL60 cells undergo apoptosis in response to RA and 4HPR,[30,31] and our FACS data are consistent with increased cell death; therefore this may be a consequence of apoptosis. However, we did not find any consensus cleavage sites for caspases 1, 3, 6, 8 and 9 within the *OVCA2* sequence, and we did not detect any increase in caspase 3 activity in response to the treatments; therefore it is unlikely that OVCA2 is a substrate for the caspases.

Further investigations are required to determine how OVCA2 is being proteolytically cleaved, how this is related to RA and 4HPR treatment and the functional consequence of this downregulation. In Calu-6 cells, which have previously been shown to undergo apoptosis in response to RA and 4HPR,[22,37] it is still not clear whether the decrease seen in OVCA2 protein levels is due to transcriptional, posttranscriptional and/or posttranslational processes. Although we did not see the degraded product in Calu-6 cells, this may be because the dead cells are removed during feeding and before harvesting.

In contrast to OVCA2, OVCA1 is not significantly affected in response to 10 μM RA or 4HPR in Calu-6 and, if anything, is slightly upregulated in response to 4HPR in A549 cells. We have shown that overexpression of OVCA1 reproducibly inhibits colony formation in several ovarian tumor cell lines and that stable expression of exogenous OVCA1 expression is difficult to obtain, which is consistent with but is not proof of a tumor suppressor function.[21] However, overexpression of OVCA2 in a variety of tumor cell lines has no obvious effects on growth (Prowse and Godwin, unpublished data). The fact that OVCA2 is downregulated in Calu-6 and HL-60 cells also suggests that OVCA2 is not likely to be a tumor suppressor.

The MRAL, which we have mapped in ovarian tumors,[20] is in fact only 20 kb, and our mapping studies indicate that there are only 3 genes in this region, *OVCA1* and *OVCA2*, which we have previously reported,[20] and *OVCA4*, which is a testis-specific gene (Godwin, unpublished data). It therefore seems likely that *OVCA1*, not *OVCA2*, is the likely tumor suppressor gene in this region. It is of interest that the Calu-6 and A549 cell lines analyzed by Liu *et al.*[22] exhibited no *OVCA1* transcript by Northern blot analysis, but our analyses do show OVCA1 mRNA and protein in these cell lines. This could be due to analyzing different subpopulations of the cell lines. Their cell lines are of interest since the lack of *OVCA1* transcript suggests that *OVCA1* could be a tumor suppressor gene involved in the development of lung tumors. Indeed, studies have shown that LOH at 17p is one of the most frequent alterations in lung cancer.[15,16,38] In addition, LOH at 17p13.3 is more frequent than at 17p13.1, where *TP53* maps, and it appeared to occur in the absence of *TP53* mutation and/or 17p13.1 dele-

tion.[15,16,38] It will be important to investigate further the role of *OVCA1* in the development of lung cancer. In addition, it will be of interest to analyze the effect that RA and 4HPR have on OVCA1 expression in breast and ovarian cell lines.

In summary, *OVCA2* is a novel gene identified on chromosome 17p13.3. *OVCA2* is composed of 2 exons: a unique exon 1 and an exon 2, which comprise part of the 3′ untranslated region of *OVCA1*. Thus, the 2 genes are overlapping, but their protein products are completely distinct. Both *OVCA1* and *OVCA2* are highly conserved, suggesting they have important roles in the cell. The homology of *OVCA2* to α-β hydrolases suggests that it may have some enzymatic activity; however, further studies are required to determine the significance of this observation. Further analysis of the function(s) of OVCA2 will help to determine the role of OVCA2 role in retinoid-induced growth arrest, differentiation and apoptosis.

ACKNOWLEDGEMENTS

A.K.G. was the recipient of National Institutes of Health grant RO1 CA-70328, United States Army Medical Research grant DAMD17-96-1-6088 and grants from the Ovarian Cancer Research Fund and the Eileen Stein Jacoby Fund. R.L.D. was the recipient of a grant from the American Cancer Society.

REFERENCES

1. Evans TR, Kaye SB, Hansen LA, Sigman CC, Andreola F, Ross SA, Kelloff GJ, De Luca LM. Retinoids: present role and future potential. Br J Cancer 1999;80:1–8.
2. Hansen LA, Sigman CC, Andreola F, Ross SA, Kelloff GJ, De Luca LM. Retinoids in chemoprevention and differentiation therapy. Carcinogenesis 2000;21:1271–9.
3. Chambon P, Hansen LA, Sigman CC, Andreola F, Ross SA, Kelloff GJ, De Luca LM. The retinoid signaling pathway: molecular and genetic analyses. Semin Cell Biol 1994;5:115–25.
4. DiSepio D, Sutter M, Johnson AT, Chandraratna RA, Nagpal S. Identification of the AP1-antagonism domain of retinoic acid receptors. Mol Cell Biol Res Commun 1999;1:7–13.
5. Nagpal S, Athanikar J, Chandraratna RA. Separation of transactivation and AP1 antagonism functions of retinoic acid receptor alpha. J Biol Chem 1995;270:923–7.
6. Salbert G, Fanjul A, Piedrafita FJ, Lu XP, Kim SJ, Tran P, Pfahl M. Retinoic acid receptors and retinoid X receptor-alpha down-regulate the transforming growth factor-beta 1 promoter by antagonizing AP-1 activity. Mol Endocrinol 1993;7:1347–56.
7. Clifford JL, Menter DG, Wang M, Lotan R, Lippman SM. Retinoid receptor-dependent and -independent effects of N-(4-hydroxyphenyl)-retinamide in F9 embryonal carcinoma cells. Cancer Res 1999;59: 14–8.
8. Kitareewan S, Spinella MJ, Allopenna J, Reczek PR, Dmitrovsky E. 4HPR triggers apoptosis but not differentiation in retinoid sensitive and resistant human embryonal carcinoma cells through an RARgamma independent pathway. Oncogene 1999;18:5747–55.
9. Sun SY, Li W, Yue P, Lippman SM, Hong WK, Lotan R. Mediation of N-(4-hydoxyphenyl)retinamide-induced apoptosis in human cancer cells by different mechanisms. Cancer Res 1999;59:2493–8.
10. Atkin NB, Baker MC. Chromosome 17p loss in carcinoma of the cervix uteri. Cancer Genet Cytogenet 1989;37:229–33.
11. Biegel JA, Burk CD, Barr FG, Emanuel BS. Evidence for a 17p tumor related locus distinct from p53 in pediatric primitive neuroectodermal tumors. Cancer Res 1992;52:3391–5.
12. Cogen PH, Daneshvar L, Metzger AK, Duyk G, Edwards MS, Sheffield VC. Involvement of multiple chromosome 17p loci in medulloblastoma tumorigenesis. Am J Hum Genet 1992;50:584–9.
13. Coles C, Thompson AM, Elder PA, Cohen BB, Mackenzie IM, Cranston G, Chetty U, Mackay J, Macdonald M, Nakamura Y, et al., Evidence implicating at least 2 genes on chromosome 17p in breast carcinogenesis. Lancet 1990;336:761–3.
14. Cornelis RS, van Vliet M, Vos CB, Cleton-Jansen AM, van de Vijver MJ, Peterse JL, Khan PM, Borresen AL, Cornelisse CJ, Devilee P. Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumors without p53 mutations. Cancer Res 1994;54: 4200–6.
15. Konishi H, Takahashi T, Kozaki K, Yatabe Y, Mitsudomi T, Fujii Y, Sugiura, T, Matsuda, H. Detailed deletion mapping suggests the involvement of a tumor suppressor gene at 17p13.3, distal to p53, in the pathogenesis of lung cancers. Oncogene 1998;17:2095–100.
16. Konishi H, Takahashi T, Kozaki K, Yatabe Y, Mitsudomi T, Fujii Y, Sugiura T, Matsuda H, Takahashi T, Takahashi T. Detailed deletion mapping suggests the involvement of a tumor suppressor gene at 17p13.3, distal to p53, in the pathogenesis of lung cancers. Oncogene 1998;17:2095–100.
17. Phillips N, Ziegle M, Saha B, Xynos F. Allelic loss on chromosome 17 in human ovarian cancer. Int J Cancer 1993;54:85–91.
18. Wiper DW, Zanotti KM, Kennedy AW, Belinson JL, Casey G. Analysis of allelic imbalance on chromosome 17p13 in stage I and stage II epithelial ovarian cancers. Gynecol Oncol 1998;71:77–82.
19. Phillips NJ, Zeigler MR, Deaven LL. A cDNA from the ovarian cancer critical region of deletion on chromosome 17p13.3. Cancer Lett 1996;102:85–90.
20. Schultz DC, Vanderveer L, Berman DB, Hamilton TC, Wong AJ, Godwin AK. Identification of two candidate tumor suppressor genes on chromosome 17p13.3. Cancer Res 1996;56:1997–2002.
21. Bruening W, Prowse AH, Schultz DC, Holgado-Madruga M, Wong A, Godwin AK. Expression of OVCA1, a candidate tumor suppressor, is reduced in tumors and inhibits growth of ovarian cancer cells. Cancer Res 1999;59:4973–83.
22. Liu G, Wu M, Levi G, Ferrari N. Down-regulation of the Diphthamide biosynthesis protein 2-like gene during retinoid-induced differentiation and apoptosis: implications against its tumor-suppressor activity. Int J Cancer 2000;88;356–62.
23. Schultz DC, Vanderveer L, Buetow KH, Boente MP, Ozols RF, Hamilton TC, Godwin AK. Characterization of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including CDKN2. Cancer Res 1995;55:2150–7.
24. Dunbrack RL. Comparative modeling of CASP3 targets using PSI-BLAST and SCWRL. Proteins (Suppl) 1999;81–7.
25. Salicioni AM, Xi M, Vanderveer LA, Balsara B, Testa JR, Dunbrack RL, Godwin AK. Identification and structural analysis of human RBM8A and RBM8B: two highly conserved RNA-binding motif proteins that interact with OVCA1, a candidate tumor suppressor. Genomics 2000;69:54–62.
26. Altschul SF, Koonin EV. Iterated profile searches with PSI-BLAST—a tool for discovery in protein databases. Trends Biochem Sci 1998;23:444–7.
27. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The protein data bank. Nucleic Acids Res 2000;28:235–42.
28. Bower MJ, Cohen FE, Dunbrack RL. Prediction of protein side-chain rotamers from a backbone-dependent rotamer library: a new homology modeling tool. J Mol Biol 1997;267:1268–82.
29. Dunbrack RL, Cohen FE. Bayesian statistical analysis of protein side-chain rotamer preferences. Protein Sci 1997;6:1661–81.
30. Delia D, Aiello A, Lombardi L, Pelicci PG, Grignani F, Formelli F, Menard S, Costa A, Veronesi U, Pierotti MA. N-(4-hydroxyphenyl)-retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. Cancer Res 1993;53: 6036–41.
31. Martin SJ, Bradley JG, Cotter TG. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin Exp Immunol 1990;79:448–53.
32. Kim KK, Song HK, Shin DH, Hwang KY, Choe S, Yoo OJ, Suh SW. Crystal structure of carboxylesterase from *Pseudomonas fluorescens*, an alpha/beta hydrolase with broad substrate specificity. Structure 1997;5:1571–84.
33. Kelley LA, MacCallum RM, Sternberg MJ. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol 2000;299:499–520.
34. Jones DT, Taylor WR, Thornton JM. A new approach to protein fold recognition. Nature 1992;358:86–9.
35. Phillips NJ, Ziegler MR, Radford DM, Fair KL, Steinbrueck T, Xynos FP, Donis-Keller H. Allelic deletion on chromosome 17p13.3 in early ovarian cancer. Cancer Res 1996;56:606–11.
36. De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 1991;5:2924–33.
37. Li Y, Dawson MI, Agadir A, Lee MO, Jong L, Hobbs PD, Zhang XK. Regulation of RAR beta expression by RAR- and RXR-selective retinoids in human lung cancer cell lines: effect on growth inhibition and apoptosis induction. Int J Cancer 1998;75:88–95.
38. Tsuchiya E, Tanigami A, Ishikawa Y, Nishida K, Hayashi M, Tokuchi Y, Hashimoto T, Okumura S, Tsuchiya S, Nakagawa K. Three new regions on chromosome 17p13.3 distal to p53 with possible tumor suppressor gene involvement in lung cancer. Jpn J Cancer Res 2000; 91:589–96.